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Cholera Toxin Indirectly Activates Human Monocyte-Derived Dendritic Cells In Vitro through the Production of Soluble Factors, Including Prostaglandin E_2 and Nitric Oxide

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Cholera toxin (CT) is a potent adjuvant that activates dendritic cells (DC) by increasing intracellular cyclic AMP (cAMP) levels. In vivo and in vitro, very small amounts of CT induce potent adjuvant effects and activate DC. We hypothesized that DC intoxicated by CT may release factors that enhance their own maturation and induce the maturation of toxin-free bystander DC. Through the use of mixed cultures and transwell cultures, we found that human monocyte-derived DC (MDDC) pulsed with CT or other cAMP-elevating agonists induce the maturation of bystander DC. Many DC agonists including CT increase the production of prostaglandin E_2 (PGE_2) and nitric oxide (NO). For this reason, we determined whether the actions of PGE_2 or NO are involved in the maturation of MDDC induced by CT or dibutyryl-cAMP (d-cAMP). We found that blocking the production of PGE_2 or blocking prostaglandin receptors inhibited MDDC maturation induced by CT and d-cAMP. Likewise, sequestering NO or blocking the downstream actions of NO resulted in the inhibition of MDDC maturation induced by CT and d-cAMP. These results indicate that endogenously produced factors including PGE_2 and NO contribute to the maturation of DC induced by CT and that these factors participate in bystander DC maturation. The results of this study may help explain why bacterial toxins that elevate cAMP are such potent adjuvants.

Understanding the intercellular and intracellular signaling processes that lead to dendritic cell (DC) maturation is important for determining how these cells initiate immune responses to foreign antigens. Monocyte-derived DC (MDDC), produced by culturing monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 6 to 7 days, are phenotypically equivalent to the immature DC that reside in peripheral tissues (45). However, it is becoming increasingly evident that the kinetics of in vivo DC differentiation from monocytes may not be reflected by the long-term in vitro culture conditions required to generate MDDC. A recent study showed that mature DC can be generated from human monocytes in as little as 48 h (14). We have studied the transition of monocytes to DC in vitro and have also generated a shortened protocol for the in vitro generation of DC. With our protocol, MDDC are matured after only 4 days of culture in the presence of GM-CSF and IL-4 (2–4). In our hands, MDDC at day 4 are more responsive to activation than MDDC at days 5 to 7.

Cholera toxin (CT) is an AB5 enterotoxin produced by *Vibrio cholerae*, the primary causative agent of the disease cholera. CT consists of a 27-kDa catalytic A domain anchored in a ring of five identical 11.7-kDa B subunits (40). The B pentamer of CT binds to GM1 gangliosides on cell membranes (28). CT has a Lys/Arg-Asp-Glu-Leu ([K/R]DEL) signal sequence at the C terminus of its A domain. This sequence is believed to target the toxin to the endoplasmic reticulum, where the A1 subunit (disguised as a misfolded host protein) is transported to the cytoplasm by the endoplasmic reticulum-associated degradation pathway (reviewed in reference 27). In the cytosol, the A1 subunit catalyzes the transfer of an ADP-ribose from NAD to stimulatory α-subunits of G proteins (Gsα). After ADP-ribosylation, Gsα binds to adenylate cyclase and constitutively activates it, leading to a sustained increase in the intracellular cyclic AMP (cAMP) concentration (12). The increased intracellular cAMP concentrations in intestinal epithelial cells are the primary cause of the massive fluid and electrolyte release associated with the disease cholera.

CT is also a powerful mucosal immunogen and adjuvant (reviewed in reference 35). When delivered mucosally, CT induces strong primary and secondary antibody responses and long-lasting immunologic memory to itself and to coadministered antigens (36, 48). In mice, antibody responses to CT and bystander antigens can last up to 2 years (36, 48). The activation/maturation of DC at sites of immunization may be responsible for the potent immunogenic and adjuvant effects of CT. In support of this, CT and the related toxins *Escherichia coli* heat-labile enterotoxin and pertussis toxin were found to induce the maturation of DC (2, 3, 21). With further study, the elevated cAMP levels induced by these toxins were found to be responsible for DC maturation (2, 3), and their enzymatic activity has been shown to play a dominant role in adjuvanticity (5, 6, 23, 34, 37).

Prostaglandins, most notably prostaglandin E_2 (PGE_2), have gained notoriety for their ability to synergize with other agonists to activate DC (42). Interestingly, CT has been shown to increase the production of PGE_2 in many cell types, including

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DC, through the release of arachidonic acid (7, 8, 11, 18, 39). Nitric oxide (NO) and superoxide anions have also recently gained attention for their ability to directly activate DC or to synergize with other agonists that activate DC (29, 41). Like prostaglandins, NO can either work directly on the cells that produce it or exert effects on neighboring cells. One of the most well-known effects of NO is the activation of guanylate cyclase (15), which results in the conversion of GTP to cyclic GMP (cGMP) and the subsequent activation of cGMP-dependent protein kinase G.

In vitro and in vivo, low concentrations of CT activate large numbers of DC. For this reason, we hypothesized that DC activated by CT may release soluble factors that enhance their own maturation and induce the maturation of toxin-free bystander DC. In support of this hypothesis, we found that CT-stimulated MDDC produce PGE_2 and NO and that these factors contribute to the maturation of the stimulated MDDC and most likely contribute to the maturation of bystander MDDC. The results of this study provide new insights into how immune responses are initiated at points of pathogen entry and may help explain the potent adjuvant effects of cholera-like enterotoxins.

MATERIALS AND METHODS

Reagents. Indomethacin, aspirin, NS-398, bromoenol lactone, aristolochic acid, LNAC (*N*-acetyl-L-cysteine), PTIO (2-phenyl-4,4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), carboxy-PTIO [2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide potassium salt), lipopolysaccharide (LPS), dibutyryl-cAMP (d-cAMP), forskolin, YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole), dibutyryl-cGMPs, and GM1 ganglioside were purchased from Sigma (St. Louis, MO). CT was purchased from List Biological Laboratories (Campbell, CA). DAF-2 (4,5-diaminofluorescein) diacetate was purchased from the Cayman Chemical Company (Ann Arbor, MI).

Cell culture medium. Medium consisted of RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine (Sigma), 1% stock nonessential amino acids (Life Technologies), 1% stock sodium pyruvate (Life Technologies), 50 μM 2-mercaptoethanol (Sigma), 50 μg/ml gentamicin (Life Technologies), and 10% fetal calf serum (Life Technologies).

Dendritic cell preparations. All human specimens were obtained under informed consent as approved by the University of Maryland Baltimore Institutional Review Board. MDDC were generated as described previously, with minor modifications (45). Human peripheral blood mononuclear cells were enriched for $CD14^+$ monocytes by negative selection (StemCell Technologies, Vancouver, Canada). The isolated monocytes were adhered to plastic and then washed with RPMI 1640 medium. The cells were cultured at 1×10^6 cells per/ml in DC culture medium supplemented with 50 ng/ml recombinant GM-CSF and 1,000 units/ml recombinant IL-4 (R&D Systems, Minneapolis, MN).

Mixed lymphocyte response. MDDC from three different donors were prepared as described above and washed three times before addition to a single donor's T cells. Naïve $CD4^+$ T cells were enriched from peripheral blood mononuclear cells by negative selection (StemCell Technologies). Naïve $CD4^+$ T cells were cultured in triplicates at 1×10^5 cells/well with 3,000 allogeneic MDDC in 96-well U-bottom plates. The cells were pulsed with 1 μCi/well [^{3}H]thymidine (Perkin-Elmer Life Sciences, Boston, MA) for the last 18 h of culture before measuring thymidine incorporation using a Packard Matrix 96 direct beta counter on the fifth day. Percent inhibition was calculated with the following formula: 100 − [(inhibited cpm − unstimulated cpm)/(uninhibited cpm − unstimulated cpm) × 100].

Flow cytometry. Cells were incubated for 30 min at 4°C with murine monoclonal antibodies specific for CD80, CD83, CD86, and HLA-DR (BD Pharmingen, San Diego, CA), washed, and then fixed with 2% paraformaldehyde for analysis using a FACScalibur flow cytometer (BD, San Jose, CA). Data analysis was carried out using FlowJo software (Tree Star Inc., San Carlos, CA).

Calculation of percent phenotypic activation and inhibition. The fraction of MDDC that increased the expression of maturation markers on the cell surface (percent activation) was calculated with FlowJo software by overlaying the histograms of treated and untreated MDDC and Overton subtraction of the curves. Percent inhibition was calculated with the following formula: $[(X - Y)/X] \times 100$, where X is the fraction of cells that increased the expression of a marker in the absence of the inhibitor and Y is the fraction of cells that increased the expression of a marker in the presence of the inhibitor.

Inhibitor concentrations. The optimal concentration of each inhibitor used was determined as follows. Baseline concentrations were obtained from previously published reports. If the inhibitor did not have adverse effects on the cells (determined by trypan blue staining and changes in forward- or side-scatter profiles) after 20 h and if it displayed significant inhibition, this concentration was used for all other experiments. However, if the inhibitor displayed weak inhibition, the concentration was increased until adverse effects were apparent on the cells. The highest concentration of the inhibitor that did not induce adverse effects on the cells was then used for all further experiments.

Tumor necrosis factor alpha (TNF-α) ELISA. Day 4 MDDC (2×10^6 cells/well in 12-well culture dishes in a total volume of 2 ml) were left untreated or were treated with 1 μg/ml of CT or 1 mM d-cAMP in the presence or absence of 1 μg/ml of LPS. Twelve hours later, supernatants were removed from the cells and analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems).

PGE_2 EIA. Day 4 MDDC (2×10^6 cells/well in 12-well culture dishes in a total volume of 2 ml) were left untreated or were treated with 1 μg/ml of CT or LPS or 1 mM d-cAMP. Twelve hours later, supernatants were removed from the cells. The supernatants were diluted 1:10 with PGE_2 enzyme immunoassay (EIA) buffer (Cayman). The diluted supernatants were analyzed by PGE_2 EIA according to the manufacturer's instructions (Cayman).

DC migration assay. DC migration was assessed using 24-well transwell plates with an 8-μm membrane (Costar, Corning, NY). MDDC (1×10^5 cells) were resuspended in 200 μl MDDC medium without GM-CSF or IL-4 and placed into the upper chamber of the transwell system. The lower chamber contained 500 μl of MDDC medium supplemented with 100 ng/ml each of macrophage inflammatory protein 3β (MIP-3β) and 6Ckine (R&D Systems). After 2 h of incubation at 37°C, the cells in the lower chamber were counted. Control cultures were kept in the absence of chemokines to assess baseline migration activity.

Statistics. P values were determined using a Student's t test.

RESULTS

Bystander maturation of MDDC. We hypothesized that DC stimulated with CT or other cAMP-elevating agonists (d-cAMP and forskolin) may release soluble factors that induce the maturation of bystander DC. Forskolin is a cell-permeable diterpenoid with adenylate cyclase-activating properties, and d-cAMP is a membrane-permeable analog of cAMP. As a first test of our hypothesis, MDDC were left unstimulated or were stimulated (incubated for 4 h with 1 mM d-cAMP and 100 μM forskolin and then washed three times to remove the agonist). These stimulated or unstimulated control MDDC (control MDDC were also washed three times) were replated at a 1:1 ratio with bystander MDDC (MDDC labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester [CFSE]) in fresh culture medium. Twenty hours after replating, the cells were stained for flow cytometry with phycoerythrin (PE)-labeled anti-CD83.

Figure 1A shows that the unstimulated and bystander cells in the control cultures remained CD83 negative, indicating that these cells retained an immature phenotype. By contrast, as shown in Fig. 1B, the stimulated MDDC and the bystander MDDC in the stimulated cultures became CD83 positive, indicating that these cells had undergone maturation. It should be noted that the concentrations of residual d-cAMP and forskolin in the cultures of the stimulated cells, after three washes, were far less than the minimum concentrations necessary to induce MDDC maturation (data not shown). Therefore, the maturation of bystander MDDC is unlikely to be due to the effects of residual d-cAMP or forskolin in the cultures.

To determine if bystander maturation is the result of soluble

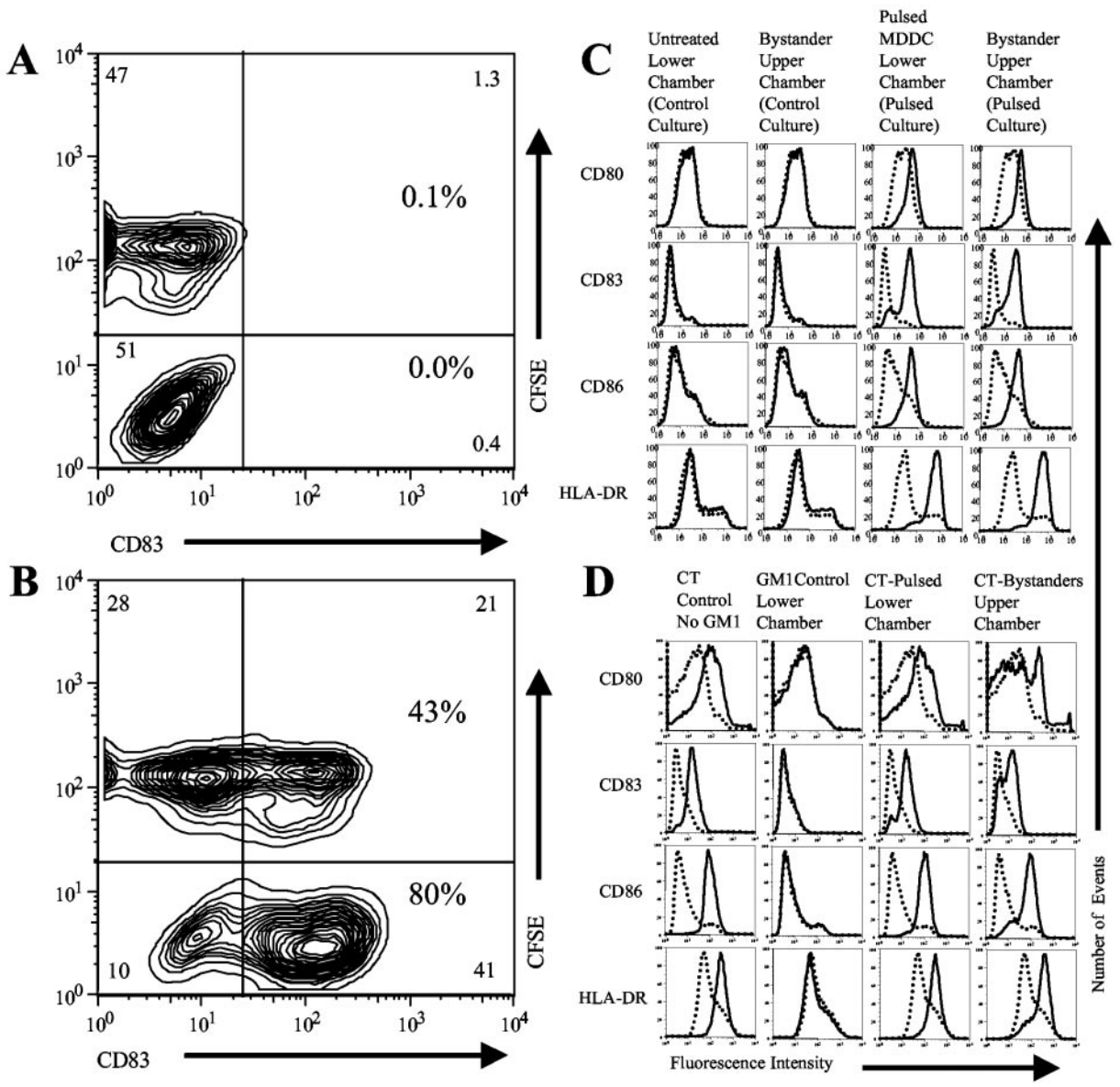

FIG. 1. Bystander maturation of MDDC induced by cAMP-elevating agonists. (A and B) Bystander maturation of MDDC in mixed cultures. MDDC were pulsed (B) or not pulsed (A) with d-cAMP and forskolin (incubated for 4 h with 1 mM d-cAMP and 100 μM forskolin and then washed three times to remove the agonist). These pulsed or unpulsed control MDDC (control MDDC were washed three times) were then replated at a 1:1 ratio with unpulsed bystander MDDC (MDDC labeled with 1 μM CFSE) in fresh culture medium. Twenty hours after replating, the cells were harvested and stained for flow cytometry with PE-labeled anti-CD83. (A) Control cultures (unpulsed MDDC mixed with CFSE-labeled bystander MDDC). (B) Pulsed cultures (pulsed MDDC mixed with CFSE-labeled bystander MDDC). Data are representative of one experiment of three performed with similar results on MDDC derived from different donors. (C) Bystander maturation of MDDC by d-cAMP and forskolin-pulsed MDDC in transwell cultures. MDDC were pulsed or not pulsed with d-cAMP and forskolin (incubated for 4 h with 1 mM d-cAMP and 100 μM forskolin and then washed three times to remove the agonist). These pulsed or unpulsed control MDDC were then replated, and untreated bystander MDDC were plated in transwell inserts (upper chambers) and inserted into the culture wells containing the pulsed or unpulsed control MDDC (bottom chamber). Twenty hours later, the cells in the upper and lower chambers were harvested and stained for flow cytometry with PE-labeled anti-CD80, fluorescein isothiocyanate (FITC)-labeled anti-CD83, CyC-labeled anti-CD86, and PE-labeled anti-HLA-DR. Cells in transwell cultures (solid histograms) are compared to untreated MDDC from separate cultures (dotted histograms). Data are representative of one experiment of three performed with similar results on MDDC derived from different donors. (D) Bystander maturation of MDDC by CT-pulsed MDDC in transwell cultures. MDDC were pulsed with CT (incubated with 5 μg/ml of CT for 10 min before the addition of 10 μg/ml of GM1). In GM control cultures, GM1 was added 10 min before CT. In CT control cultures, no GM1 was added. Thirty minutes after the addition of CT, untreated bystander MDDC in transwell inserts (upper chambers) were inserted into the culture wells containing the CT-treated MDDC (lower chambers). Twenty hours later, the cells in the upper and lower chambers were harvested and stained for flow cytometry with PE-labeled anti-CD80, FITC-labeled anti-CD83, FITC-labeled anti-CD86, and PE-labeled anti-HLA-DR. Cells in transwell cultures (solid histograms) are compared to untreated MDDC from separate cultures (dotted histograms). Data are representative of one experiment of three performed with similar results on MDDC derived from different donors.

factors, we designed experiments utilizing transwell culture dishes. MDDC were left unstimulated or were stimulated with d-cAMP and forskolin as described above. These stimulated or unstimulated control MDDC were replated in the lower chambers of transwell culture dishes. Bystander MDDC were placed in the upper transwell inserts. Twenty hours later, the cells in the upper and lower chambers were assayed by flow cytometry. Figure 1C shows that the bystander MDDC in the stimulated cultures (upper chamber) displayed phenotypic maturation. These results indicate that bystander maturation is due to the effects of soluble factors.

Initially, CT-stimulated MDDC were not tested for the ability to induce bystander maturation, as CT cannot be completely washed away from the stimulated cells. However, the effects of CT on MDDC can be completely blocked by the addition of soluble GM1 to the culture medium. In this regard, adding 10 μg/ml of GM1 to a culture of MDDC 10 min before the addition of 5 μg/ml of CT completely blocks the maturation of the MDDC induced by CT but not by LPS (GM1 control cells) (data not shown). By contrast, adding 10 μg/ml of GM1 to a culture of MDDC 10 min after the addition of 5 μg/ml of CT has no effect on MDDC maturation (CT-pulsed cells) (data not shown).

For these experiments, MDDC in the bottom chambers of transwell plates were pulsed with CT (incubated with 5 μg/ml of CT for 10 min before the addition of 10 μg/ml of GM1). Thirty minutes after the addition of CT, bystander MDDC were added to the upper chambers of the transwell cultures. In the GM1 control cultures, 10 μg/ml of GM1 was added 10 min before the addition of 5 μg/ml of CT. CT control cells were incubated with CT in the absence of GM1. Twenty hours later, the cells in the upper and lower chambers were harvested and assayed by flow cytometry and a mixed-lymphocyte reaction (MLR). Figure 1D shows that GM1 completely inhibited the ability of CT to induce the phenotypic maturation of MDDC in the GM1 control cultures. By contrast, in the pulsed cultures, the CT-pulsed MDDC (lower chamber) and the bystander MDDC (upper chamber) displayed phenotypic maturation. In addition, Fig. 2A shows that CT-pulsed MDDC and CT bystander MDDC from the pulsed cultures were equally potent antigen-presenting cells (APC) in an MLR, indicating that both populations were functionally mature. The increases in T-cell proliferation induced by the stimulated and bystander cells are statistically significant ($P < 0.05$).

Cholera toxin-stimulated and bystander MDDC display similar migrations to CCR7-binding chemokines. The maturation of DC has been reported to be associated with a switch in chemokine receptor expression from inflammatory to constitutive, such that CCR7 is up-regulated following maturation (16, 17, 19). CCR7 mediates DC migration by chemokines preferentially expressed in lymph nodes, such as 6Ckine and MIP-3β.

Transwell cultures were established using d-cAMP and forskolin-pulsed or CT-pulsed MDDC and bystanders, as described above. As an additional control, MDDC were matured in the presence of 100 ng/ml of LPS. Forty hours after the initiation of the cultures, the agonist-pulsed cells from the lower chambers and the bystander cells from the upper chambers were harvested, washed, and used in migration assays as follows. A total of 1×10^5 cells were placed into the upper chamber of a transwell system where the lower chamber contained medium supplemented with 100 ng/ml each of MIP-3β and 6Ckine. Control cultures were set up in the absence of chemokines to assess random migration activity. After 2 h of incubation at 37°C, the migrated MDDC in the lower chambers were harvested and counted.

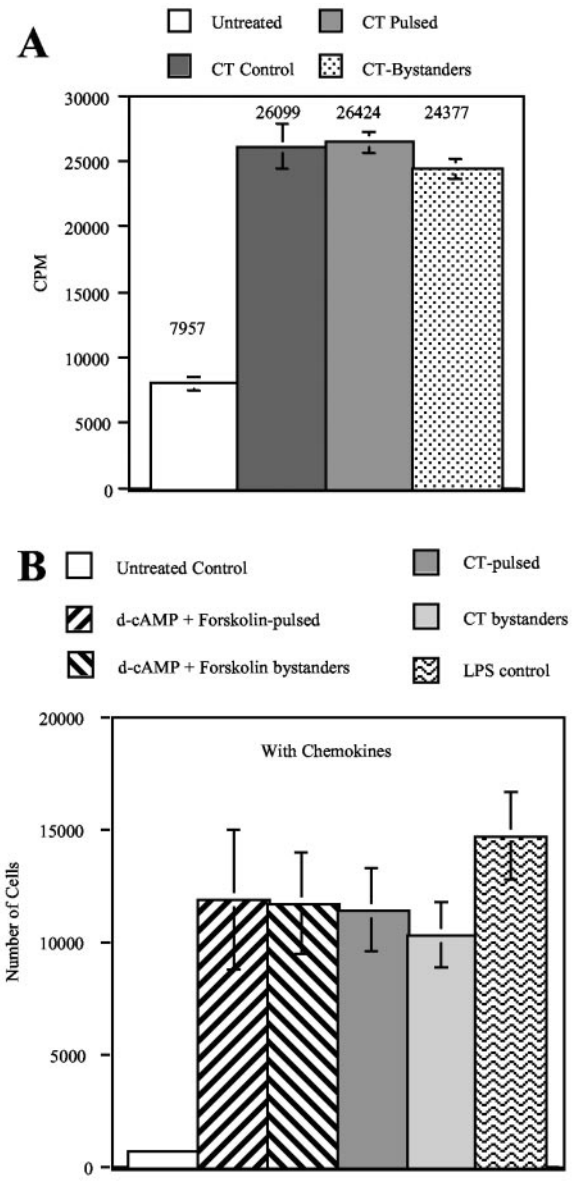

FIG. 2. Bystander MDDC are functionally mature. (A) Bystander MDDC induce robust T-cell proliferation. MDDC prepared in Fig. 1D were used in an MLR as described in Materials and Methods. Data shown are the means and standard errors from cells derived from three different donors. (B) Migration of MDDC in response to MIP-3β and 6Ckine. MDDC (1×10^5 cells) were placed into the upper chamber of the transwell system. The lower chamber contained medium supplemented with 100 ng/ml each of MIP-3β and 6Ckine. After 2 h of incubation at 37°C, the cells in the lower chamber were counted. Control cultures were kept in the absence of chemokines to assess random migration activity. Data shown are the means and standard errors of the means from cells derived from three different donors.

Figure 2B shows that MDDC stimulated by d-cAMP and

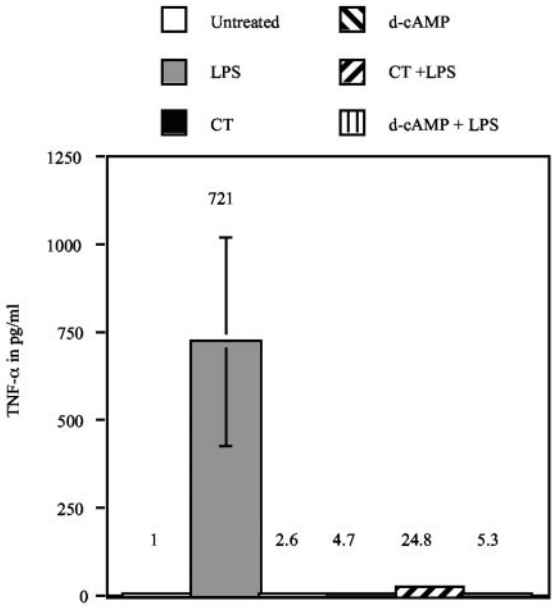

FIG. 3. TNF-α is not responsible for bystander maturation. Day 4 MDDC were left untreated or were treated with 1 μg/ml CT or 1 mM d-cAMP in the presence or absence of 1 μg/ml LPS. After 12 h of culture, TNF-α concentrations in the supernatants were determined by ELISA. Data shown are the means and standard errors of the means from cells derived from at least three different donors.

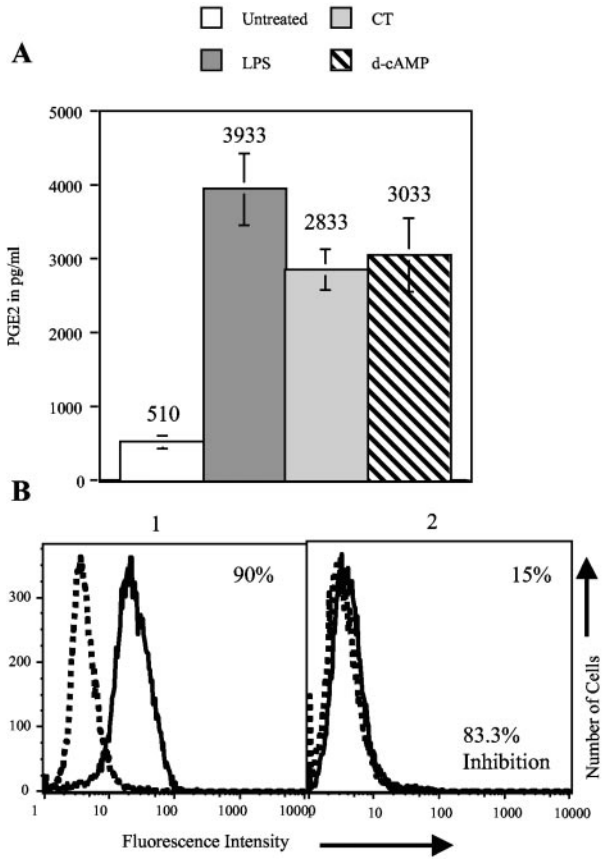

FIG. 4. PGE_2 production by MDDC. (A) MDDC were treated with 1 μg/ml CT or LPS or 1 mM d-cAMP. Twelve hours later, supernatants were removed, and PGE_2 concentrations in the supernatants were measured by EIA. Data were obtained from three experiments performed on MDDC derived from different donors. The means and standard errors of the means are shown. (B) Example calculation of percent inhibition. The formula used to calculate percent inhibition is $[(X - Y)/X] \times 100$, where X is the fraction of cells that increased the expression of a marker in the absence of the inhibitor and Y is the fraction of cells that increased the expression of a marker in the presence of the inhibitor. Panel 1 shows CD83 expression in the absence of an inhibitor, and panel 2 shows CD83 expression in the presence of an inhibitor. X is 90, Y is 15, and the equation $[(90 - 15) \times 100]/90$ equals 83.3% inhibition.

forskolin or CT showed similar migration activity to that of LPS-matured MDDC, a level far above that of the immature MDDC in the control cultures. Likewise, the bystander MDDC in the stimulated cultures showed similar migration activity to that of LPS-matured MDDC. The increases in migration of the stimulated and bystander cells are statistically significant ($P < 0.05$). For all of the tested MDDC, in the absence of chemokines, less than 1,000 cells migrated to the lower chambers (data not shown). These results, in addition to the results described above, indicate that MDDC stimulated to maturity by cAMP-elevating agonists release soluble factors that induce the full maturation of bystander MDDC not stimulated by the agonist.

MDDC incubated with CT or d-cAMP dominantly inhibit the production of TNF-α. The most obvious candidate factors responsible for the bystander maturation described above are inflammatory cytokines. In this regard, TNF-α activates DC and is routinely used in cocktails for the maturation of MDDC used in clinical trials. TNF-α has also been shown to synergize with PGE_2 for more potent activation of DC. However, it has been shown that cAMP-elevating agonists such as CT dominantly inhibit the production of inflammatory cytokines (2, 3, 21). For these reasons, we measured TNF-α concentrations in the supernatants of MDDC cultures stimulated with CT or d-cAMP. Day 4 MDDC were left untreated or were treated with 1 μg/ml CT or 1 mM d-cAMP in the presence or absence of 1 μg/ml LPS. After 12 h in culture, TNF-α concentrations in the supernatants were determined by ELISA.

Figure 3 shows that MDDC incubated with CT or d-cAMP do not produce TNF-α. In addition, these cAMP-elevating agents dominantly inhibit the production of this cytokine induced by LPS. The increase in TNF-α production by LPS-stimulated cells over that of untreated cells is statistically significant ($P < 0.05$). The differences in TNF-α production between CT- or d-cAMP-stimulated cells in the presence or absence of LPS and untreated cells are not statistically significant ($P > 0.05$). Similar results have been found with other cytokines such as IL-1, IL-6, and IL-12 (2, 3, 21). These results indicate that inflammatory cytokines such as TNF-α are unlikely to be responsible for the bystander maturation described above.

Products of the COX enzymes contribute to the maturation of MDDC induced by CT and d-cAMP. PGE_2 has been shown to influence DC maturation. For this reason, we determined whether PGE_2 or other products of the cycloxygenase (COX) enzymes contribute to the maturation of MDDC induced by elevated cAMP levels and/or contribute to bystander maturation. The effects of exogenous PGE_2 on DC function have been

TABLE 1. Prostaglandin production contributes to the maturation of MDDC induced by cAMP-elevating agonists[a]

Inhibitor	Concn	Marker	Avg % inhibition (SEM)		
			LPS	CT	d-cAMP
Aspirin	10 μg/ml	CD80	13.4 (5.1)	11.7 (9.5)	16.7 (9.1)
		CD83	18.3 (7.6)	15.0 (6.1)	19.7 (3.3)
		CD86	23.1 (6.7)	17.3 (13.4)	18.0 (8.7)
		HLA-DR	12.0 (3.4)	16.7 (8.0)	22.3 (4.8)
		MLR	NT	NT	NT
NS-398	10 μM	CD80	0.7 (0.5)	11.3 (6.3)	18.3 (8.0)
		CD83	5.3 (2.4)	11.7 (6.2)	17.0 (5.8)
		CD86	3.7 (1.9)	15.3 (9.1)	9.0 (2.4)
		HLA-DR	3.3 (2.7)	14.7 (7.0)	15.3 (4.0)
		MLR	NT	NT	NT
Indomethacin	10 μg/ml	CD80	60.4 (8.8)	83.1 (3.1)	59.7 (6.7)
		CD83	39.1 (4.6)	43.5 (5.2)	39.8 (3.2)
		CD86	58.4 (6.7)	54.8 (12.5)	60.2 (6.3)
		HLA-DR	63.1 (3.7)	71.2 (1.3)	60.5 (3.5)
		MLR	26.5 (2.5)	25.7 (4.0)	23.5 (4.1)
Bromoenol lactone	10 μM	CD80	67.4 (1.8)	55.1 (10.0)	55.8 (5.0)
		CD83	70.5 (5.9)	67.7 (5.2)	52.9 (2.7)
		CD86	67.3 (10.5)	58.9 (1.9)	54.9 (5.6)
		HLA-DR	52.7 (7.7)	46.9 (7.2)	46.7 (7.7)
		MLR	24.6 (2.6)	26.2 (5.0)	32.1 (2.7)
Aristolochic acid	100 μM	CD80	37.1 (1.5)	39.1 (9.0)	51.7 (5.1)
		CD83	37.6 (4.4)	50.8 (3.4)	32.8 (6.9)
		CD86	35.9 (4.1)	44.4 (8.7)	47.4 (4.7)
		HLA-DR	5.3 (1.0)	19.5 (7.7)	23.2 (2.9)
		MLR	NT	NT	NT
AH-6809	100 μM	CD80	75.9 (7.5)	76.4 (6.0)	75.3 (9.4)
		CD83	78.4 (7.8)	66.6 (14.8)	62.5 (20.3)
		CD86	80.7 (8.2)	70.2 (6.3)	50.7 (21.7)
		HLA-DR	73.5 (10.9)	64.6 (12.1)	57.1 (20.0)
		MLR	15.0 (1.9)	22.7 (3.0)	28.7 (6.1)

[a] MDDC were incubated with 0.5 μg/ml of LPS or CT or 500 μM d-cAMP with or without a prior 1-h incubation with the indicated concentrations of the indicated inhibitors for 20 h. The cells were then harvested and stained for four-color flow cytometry with PE-labeled anti-CD80, FITC-labeled anti-CD83, Cy-Chrome-labeled anti-CD86, and APC-labeled anti-HLA-DR. The MDDC were also tested in an MLR. The percent inhibition (averages are shown) was calculated as described in Materials and Methods. For reagents suspended in dimethyl sulfoxide (DMSO), control cultures were incubated with an equal volume of DMSO. DMSO at the concentrations used does not activate or inhibit the maturation of these cells (data not shown). The results are the means and standard errors of the means of at least three experiments performed on cells from different donors. NT, not tested.

extensively studied (for a review, see reference 38). The effects of endogenous PGE_2 production on DC function are not as well studied but have been implicated in DC chemotaxis (46) and maturation (50).

First, we tested PGE_2 production by stimulated MDDC. Day 4 MDDC were treated with 1 μg/ml of CT, 1 mM d-cAMP, or 1 μg/ml LPS (as a control). Twelve hours later, supernatants were subjected to PGE_2 EIA. Figure 4A shows that CT and d-cAMP as well as LPS increased PGE_2 production by MDDC. The increases in PGE_2 production by the stimulated cells are statistically significant ($P < 0.05$). Next, we determined whether prostaglandin production contributes to the maturation of MDDC induced by these agonists. To determine this, we incubated MDDC with these agonists in the presence of the COX-1-specific inhibitor aspirin (22) or the COX-2-specific inhibitor NS-398 (20). MDDC were incubated with 0.5 μg/ml CT, 500 μM d-cAMP, or 0.5 μg/ml LPS (as a control) with or without a prior 1-h incubation with 50 μg/ml of aspirin or 1 μg/ml of NS-398. Twenty hours later, the cells were assayed by flow cytometry. The percent inhibition was calculated as described in Materials and Methods and is illustrated in Fig. 4B.

Table 1 shows that the phenotypic maturation of the MDDC induced by CT, d-cAMP, or LPS was weakly inhibited by aspirin or NS-398. For this reason, we determined whether inhibiting both isoforms of COX induces greater inhibition. MDDC were incubated with the agonists with or without a prior 1-h incubation with 10 μg/ml of the COX-1 and COX-2 inhibitor indomethacin (32). Twenty hours later, the cells were assayed for phenotypic maturation and for functional maturation in an MLR. Table 1 shows that indomethacin strongly inhibits the phenotypic maturation of MDDC while it moderately inhibits the functional maturation of the same cells induced by both agonists. It is not surprising that the inhibition of functional maturation is not as potent as the inhibition of phenotypic maturation. In this regard, only a small percentage of a population of MDDC needs to be phenotypically mature for that population to display near-maximal functional maturation in T-cell proliferation assays. It should also be noted

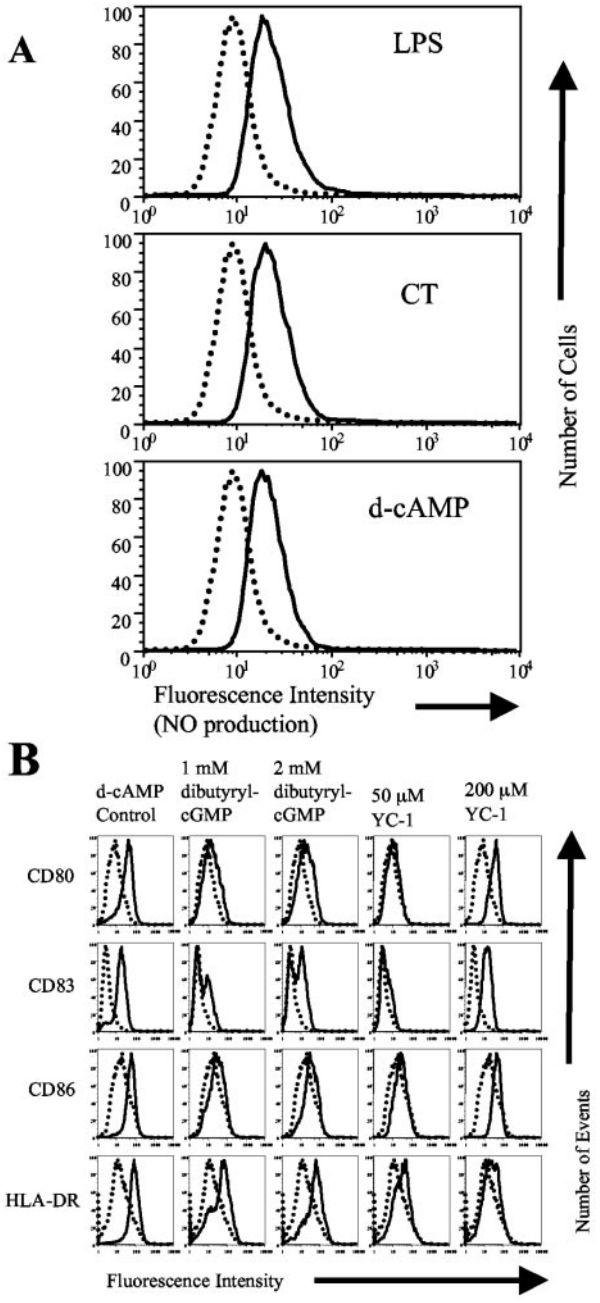

FIG. 5. Nitric oxide contributes to the maturation of MDDC induced by cAMP-elevating agonists through the elevation of cGMP. (A) Nitric oxide production by MDDC. MDDC were incubated with 10 μM DAF-2 diacetate with or without 1 μg/ml LPS, 1 μg/ml CT, or 1 mM d-cAMP. Twenty hours later, the cells were assayed by flow cytometry. Stimulated MDDC (solid histograms) are compared to untreated MDDC (dotted histograms). Data are representative of one experiment of three performed with similar results on MDDC derived from different donors. (B) Dibutyryl-cGMP and YC-1 activate MDDC to mature. Cell surface expression of the indicated markers on untreated MDDC (dotted histograms) or stimulated MDDC (solid histograms) is shown. MDDC were incubated with 1 mM d-cAMP (as a control) or the indicated concentrations of dibutyryl-cGMP or YC-1 for 20 h. Cells were then harvested and stained for four-color flow cytometry with PE-labeled anti-CD80, FITC-labeled anti-CD83, CyChrome-labeled anti-CD86, and APC-labeled anti-HLA-DR. Data are representative of one experiment of three performed with similar results on MDDC derived from different donors.

that PGE_2 is only one of many products produced by the COX enzymes. Therefore, it is possible that other products of the COX enzymes also contribute to MDDC maturation.

To verify that products of the COX enzymes contribute to the maturation of MDDC induced by elevated cAMP levels, we incubated MDDC with CT, d-cAMP, or LPS in the presence or absence of the phospholipase A2 inhibitor bromoenol lactone (26) (10 μM) or aristolochic acid (44) (100 μM) or the prostaglandin D series (DP), prostaglandin E series 1 (EP_1), and EP_2 receptor antagonist AH-6809 (51) (100 μM). Phospholipase A2 is upstream of the COX enzymes in the prostaglandin production pathway, and blocking DP, EP_1, and EP_2 receptors blocks the activity of prostaglandins on target cells. Table 1 shows that all three inhibitors strongly inhibit the phenotypic maturation of MDDC, while bromoenol lactone and AH-6809 moderately inhibit the functional maturation of MDDC. These results further indicate that products of the COX enzymes, including PGE_2, contribute to the maturation of MDDC induced by CT, d-cAMP, and LPS.

Nitric oxide contributes to the maturation of MDDC induced by CT and d-cAMP through the activation of guanylate cyclase. Elevated cAMP levels increase the expression of inducible NO synthase and increase the production of NO in many cell types (9, 13, 33). In addition, a possible role for NO in DC maturation is indicated by the ability of an NO-scavenging antioxidant to inhibit LPS-induced maturation of DC (49). For these reasons, we determined NO production by individual cells using DAF-2 diacetate, a sensitive and specific fluorescent indicator for the detection of NO (25, 31). MDDC were incubated for 20 h with 10 μM DAF-2 diacetate with or without 1 μg/ml CT, 1 mM d-cAMP, or 1 μg/ml LPS (as a control) and then analyzed by flow cytometry. Figure 5A shows that the mean channel fluorescence of MDDC incubated with DAF-2 increases when they are coincubated with CT, d-cAMP, or LPS. These results indicate that MDDC produce NO when stimulated with CT, d-cAMP, or LPS.

To test whether NO or other radicals are involved in MDDC maturation, we used radical scavengers to inhibit the effects of NO. LNAC is an agent that increases cellular pools of free radical scavengers, PTIO is a stable radical scavenger without the effect of NO synthase, and carboxy-PTIO is a scavenger that reacts with NO to form derivatives which inhibit NO synthase. For these experiments, MDDC were incubated with 0.5 μg/ml CT, 500 μM d-cAMP, or 0.5 μg/ml LPS (as a control) with or without a prior 1-h incubation with 10 μM LNAC (43), 500 μM PTIO, or 500 μM carboxy-PTIO (1). After 20 h, the cells were assayed by flow cytometry and an MLR. Table 2 shows that all three agents strongly inhibit the phenotypic maturation of MDDC while moderately inhibiting the functional maturation of MDDC, indicating that NO and/or other radicals contribute to the maturation of MDDC induced by these agonists.

To determine if NO directly activates DC, we treated MDDC with the NO donors 3-[(±)-(*E*)-ethyl-2′-[(*E*)-hydroxyimino]-5-nitro-3-hexene carbanoyl]-pyridine (NOR-4) and 3-morpholino-sydnonimine (SIN-1). We found that neither 1 mM NOR-4 nor 1 mM SIN-1 alone or together increased the expression of any maturation markers tested (data not shown). As we could not determine whether the failure of the NO donors to activate MDDC was due to an inability of NO to

TABLE 2. Nitric oxide production contributes to the maturation of MDDC induced by cAMP-elevating agonists[a]

Inhibitor	Concn (μM)	Marker	Avg % inhibition (SEM)		
			LPS	CT	d-cAMP
LNAC	10	CD80	57.6 (14.1)	59.2 (12.5)	83.7 (3.6)
		CD83	58.0 (11.8)	60.9 (16.5)	87.5 (0.4)
		CD86	57.7 (18.9)	53.2 (9.7)	86.5 (1.6)
		HLA-DR	59.2 (21.4)	52.1 (15.3)	83.2 (6.3)
		MLR	15.2 (1.5)	14.6 (2.3)	19.8 (3.9)
PTIO	500	CD80	53.4 (11.4)	65.0 (7.8)	58.7 (9.2)
		CD83	45.9 (8.3)	65.2 (7.9)	59.1 (10.9)
		CD86	64.1 (14.0)	56.2 (7.2)	44.4 (11.1)
		HLA-DR	41.3 (17.7)	49.6 (8.5)	36.5 (8.0)
		MLR	21.1 (3.5)	20.1 (1.9)	17.6 (1.8)
Carboxy-PTIO	500	CD80	83.3 (9.1)	98.7 (1.1)	98.5 (1.2)
		CD83	82.4 (7.3)	94.0 (2.8)	96.8 (1.6)
		CD86	82.7 (6.2)	87.9 (4.5)	92.8 (2.8)
		HLA-DR	81.3 (8.7)	76.7 (5.8)	89.1 (4.9)
		MLR	NT	NT	NT
ODQ	100	CD80	54.7 (5.8)	98.4 (0.5)	86.7 (5.5)
		CD83	78.0 (7.7)	94.4 (1.4)	84.1 (0.6)
		CD86	54.3 (6.6)	81.6 (1.0)	70.2 (10.9)
		HLA-DR	25.4 (6.1)	58.9 (2.1)	49.4 (8.7)
		MLR	20.5 (4.8)	19.7 (6.2)	17.5 (3.9)
KT5823	8	CD80	78.7 (2.7)	80.6 (8.0)	93.2 (2.7)
		CD83	72.5 (5.4)	73.7 (10.4)	92.9 (3.6)
		CD86	76.7 (5.5)	48.2 (8.6)	66.9 (11.8)
		HLA-DR	72.1 (4.8)	64.5 (5.9)	53.9 (15.3)
		MLR	18.9 (6.6)	21.8 (4.2)	27.3 (3.6)

[a] MDDC were incubated with 0.5 μg/ml LPS or CT or 500 μM d-cAMP with or without a prior 1-h incubation with the indicated concentrations of the indicated inhibitors for 20 h. The cells were then harvested and stained for four-color flow cytometry with PE-labeled anti-CD80, FITC-labeled anti-CD83, Cy-Chrome-labeled anti-CD86, and APC-labeled anti-HLA-DR. The MDDC were also tested in an MLR. The percent inhibition (averages are shown) was calculated as described in Materials and Methods. For reagents suspended in DMSO, control cultures were incubated with an equal volume of DMSO. DMSO at the concentrations used does not activate or inhibit the maturation of these cells (data not shown). The results are the means and standard errors of the means of at least three experiments performed on cells from different donors. NT, not tested.

activate the cells or a failure to mimic the type of NO production (time of production and amount of production) by the stimulated MDDC, we determined if a downstream signaling pathway activated by NO is involved in MDDC maturation.

One of the key downstream signaling pathways activated by NO is the guanylate cyclase/cGMP pathway. Therefore, we determined whether increased intracellular levels of cGMP activate MDDC. MDDC were incubated for 20 h with increasing concentrations of the membrane-permeable analog of cGMP, dibutyryl-cGMP, or the guanylate cyclase activator YC-1 (30). Twenty hours later, the cells were assayed by flow cytometry. Figure 5B shows that dibutyryl-cGMP and YC-1 induce phenotypic MDDC maturation in a dose-dependent manner. These results demonstrate that signaling through guanylate cyclase, a pathway known to be activated by NO, induces the phenotypic maturation of MDDC.

To test whether cGMP production by guanylate cyclase contributes to the maturation of MDDC induced by CT, d-cAMP, or LPS, we incubated MDDC for 20 h with these agonists in the presence or absence of the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a1]quinoxalin-1-one (ODQ) (10) (100 μM) or the protein kinase G inhibitor KT5823 (24) (8 μM). Table 2 shows that ODQ and KT5823 strongly inhibit the phenotypic maturation and moderately inhibit the functional maturation of MDDC induced by these agonists. Together, these results indicate that induction of guanylate cyclase, possibly by NO, contributes to the maturation of MDDC induced by elevated cAMP levels and LPS.

PGE_2 and dibutyryl-cGMP synergize for the maturation of MDDC. The above-described results indicate that products of the COX enzymes and NO contribute to the maturation of MDDC and most likely contribute to the maturation of bystander MDDC. However, the potency of either factor alone is not able to fully explain the observed bystander maturation. For this reason, we determined whether PGE_2 and elevated cGMP levels (dibutyryl-cGMP) synergize for the activation of MDDC. MDDC were incubated with 1 μg/ml of CT (as a control) or suboptimal doses of PGE_2 (1 μM) and dibutyryl-cGMP (250 μM), alone or together. Twenty hours later, the cells were analyzed by flow cytometry. Figure 6 shows that PGE_2 and dibutyryl-cGMP synergize for the phenotypic maturation of MDDC.

DISCUSSION

CT and d-cAMP activate cAMP-responsive intracellular pathways while LPS activates other intracellular pathways. Surprisingly, our data show that the maturation of MDDC induced by all of these agonists is blocked by inhibiting the production of prostaglandins or NO or by inhibiting their ef-

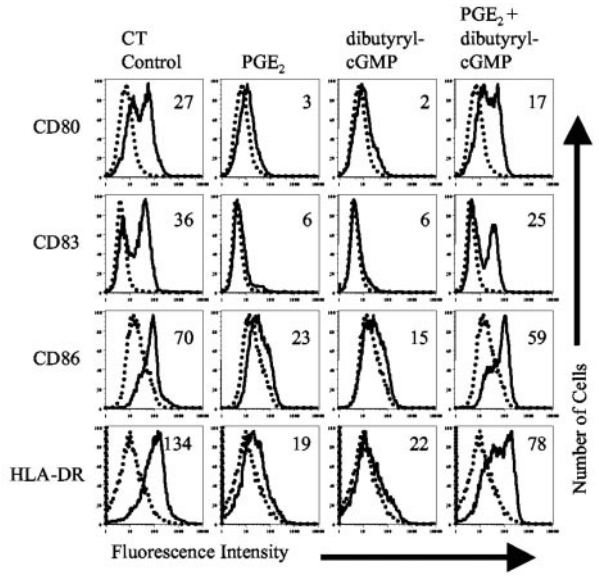

FIG. 6. Synergy between PGE_2 and dibutyryl-cGMP. Cell surface expression of the indicated markers on untreated MDDC (dotted histograms) or MDDC treated with the indicated agonist (solid histograms) is shown. MDDC were incubated with 1 μg/ml of CT (as a control) or 1 μM PGE_2 or 250 μM dibutyryl-cGMP alone or together for 20 h. The cells were then harvested and stained for four-color flow cytometry with PE-labeled anti-CD80, FITC-labeled anti-CD83, CyChrome-labeled anti-CD86, and APC-labeled anti-HLA-DR. Numbers in histograms indicate the increase in mean fluorescence of treated MDDC over untreated MDDC. Data are representative of one experiment of three performed with similar results on MDDC derived from different donors.

fects on target cells. Therefore, our data indicate that the intracellular signaling pathways activated by these agonists are not sufficient to induce the maturation of the MDDC. There are two possible explanations for these results. First, the maturation of MDDC induced by these agonists may be the result of synergy between signal transduction pathways directly activated by the agonists and pathways activated by the soluble factors produced by the stimulated MDDC. Alternatively, the maturation induced by these agonists may entirely be the result of the actions of the soluble factors. In this regard, the only role in MDDC maturation played by the signaling pathways activated directly by cAMP may be to induce the production of the soluble factors. Indeed, the ability of these soluble factors to induce the maturation of bystander MDDC demonstrates that these factors can induce MDDC maturation without synergy from the cAMP-elevating agonists. Unfortunately, the data generated in this study are not sufficient to determine which of the above-described possibilities is correct.

The data presented in this study indicate that human DC stimulated to mature by cAMP-elevating agonists release soluble factors that induce the maturation of human bystander DC not stimulated by the agonist. A separate study showed a similar phenomenon in the mouse (47). In this regard, wild-type mouse bone marrow-derived DC activated by LPS can induce the maturation of bystander $TLR4^{-/-}$ DC (47). In addition, it was shown using bone marrow chimeras that bystander maturation occurs in vivo using Toll-like receptor (TLR) agonists (47). That study found that although the bystander DC displayed all of the characteristics of fully mature DC, they failed to produce IL-12 and expanded T cells that did not display a Th1 or Th2 phenotype. Those authors hypothesized that the bystander DC may induce T cells with a regulatory phenotype that help control the immune response (47).

We have a slightly different interpretation of the data (47). Although it has been published that MDDC matured by CT expand T cells with a Th2 phenotype (21), in our hands, CT-matured DC consistently generate T cells that fail to produce Th1 or Th2 effector cytokines. Therefore, in our hands, DC matured with CT strongly resemble bystander DC matured by factors released from DC stimulated with cAMP-elevating agonists or TLR agonists. In contrast to generating tolerance, CT is known to be a potent adjuvant. Therefore, we believe that bystander DC do not exclusively expand T cells with a regulatory phenotype but rather expand effector T cells with an undetermined phenotype.

Although the other study (47) convincingly showed bystander maturation using TLR agonists in the mouse, no experiments were undertaken to determine the nature of the soluble factors involved (47). Those authors hypothesized that inflammatory mediators including inflammatory cytokines such as alpha/beta interferon are responsible for the observed bystander maturation (47). This is supported by the fact that cytokines such as TNF-α induce the maturation of DC and are routinely used in maturation cocktails for the maturation of MDDC used in clinical trials. However, DC matured by inflammatory cytokines generally produce IL-12 and expand T cells with a Th1 phenotype. Therefore, it is difficult to reconcile how bystander DC matured by inflammatory cytokines would fail to produce IL-12 and expand T cells with a regulatory phenotype.

Our data indicate that the bystander maturation may be the result of factors that are not proteins. In this regard, unlike TLR agonists, it has been shown that cAMP-elevating agonists dominantly inhibit the production of inflammatory cytokines (2, 3, 21). Therefore, the soluble mediators that are responsible for bystander maturation are more likely factors such as prostaglandins and NO that activate DC without inducing cytokine production. The full elucidation of the factors involved in this bystander phenomenon should provide valuable insights into how immune responses are initiated at points of pathogen entry.

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