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Crosstalk Between PKA and Epac Regulates the Phenotypic Maturation and Function of Human Dendritic Cells

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The cAMP-dependent signaling pathways that orchestrate dendritic cell (DC) maturation remain to be defined in detail. Although cAMP was previously thought to signal exclusively through protein kinase A (PKA), it is now clear that cAMP also activates exchange protein activated by cAMP (Epac), a second major cAMP effector. Whether cAMP signaling via PKA is sufficient to drive DC maturation or whether Epac plays a role has not been examined. In this study, we used cAMP analogs to selectively activate PKA or Epac in human monocyte-derived DCs and examined the effect of these signaling pathways on several hallmarks of DC maturation. We show that PKA activation induces DC maturation as evidenced by the increased cell-surface expression of MHC class II, costimulatory molecules, and the maturation marker CD83. PKA activation also reduces DC endocytosis and stimulates chemotaxis to the lymph node-associated chemokines CXCL12 and CCL21. Although PKA signaling largely suppresses cytokine production, the net effect of PKA activation translates to enhanced DC activation of allogeneic T cells. In contrast to the stimulatory effects of PKA, Epac signaling has no effect on DC maturation or function. Rather, Epac suppresses the effects of PKA when both pathways are activated simultaneously. These data reveal a previously unrecognized crosstalk between the PKA and Epac signaling pathways in DCs and raise the possibility that therapeutics targeting PKA may generate immunogenic DCs, whereas those that activate Epac may produce tolerogenic DCs capable of attenuating allergic or autoimmune disease. The Journal of Immunology, 2010, 185: 3227–3238.
a role, and if so, whether crosstalk between the PKA and Epac signaling pathways controls this process.

In this study, we addressed the hypothesis that cAMP activation of Epac plays a role in regulating the maturation and function of human monocyte-derived DCs. Because cAMP binds to both PKA and Epac with the same affinity (23), differentiating between the roles of PKA and Epac in cAMP-dependent cellular processes has been difficult. The recent synthesis and characterization of cAMP analogs that selectively bind and activate either PKA or Epac have now made it possible to discriminate between the two signaling pathways (24). We used highly selective cAMP analogs to examine the effect of PKA and Epac signaling on several hallmark of DC maturation including the upregulation of MHC class II and costimulatory molecules, chemotaxis to lymph node-associated chemokines, downregulation of endocytosis, changes in cytokine expression, and T cell activation. We also analyzed the effect of PKA and Epac signaling on two forms of nondirected DC migration: migration in the absence of chemical cues (random migration) and migration in symmetrical concentrations of chemotactants (chemokinesis). Our data reveal a previously unrecognized cross-talk between the PKA and Epac signaling pathways in DCs. The results of this study suggest that elucidating the points of interaction between the PKA and Epac signaling pathways will be critical for understanding how cAMP signaling is integrated in DCs to affect the initiation or inhibition of T cell-mediated immune responses in vivo.

Materials and Methods

Reagents and Abs

CT was obtained from Calbiochem (San Diego, CA). Escherichia coli 026: B6 LPS (gamma-irradiated; total impurities <5% protein) and FITC-dextran (40,000 Da) were from Sigma-Aldrich (St. Louis, MI). 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Alexis Biochemicals (Farmingdale, NY). N6-benzoyladenosine-3',5'- cyclic monophosphosphate (6-Bnz-cAMP; an inefficient Epac activator and a full PKA activator) (25) was from Sigma-Aldrich, and 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (O-Me-cAMP; the combination of 8-4cPTP and 2'-O-methyl derivatives in this cAMP analog improved the Epac/PKA binding selectivity ~3 orders of magnitude) (25) was from Biolomol Life Science Institute (Bremen, Germany). 6-Bnz-cAMP was dissolved in water, and O-Me-cAMP was dissolved in DMSO. Dibutyryl cAMP (db-cAMP) was purchased from R&D Systems. Recombinant human Epac2 (Ser3) and CREB rabbit Abs were from Cell Signaling Technology (Danvers, MA). The goat anti-rabbit IgG Ab conjugated to HRP was from Sigma-Aldrich, and the goat anti-mouse IgG conjugated to FITC was from R&D Systems. Recombinant human GM-CSF (leukine) was from Berlex Laboratories (Montville, NJ). RPMI 1640, FBS, penicillin, streptomycin sulfate, and amphotericin B were from Invitrogen (Carlsbad, CA). The following FITC-conjugated mouse Abs were purchased from BD Pharmingen (San Jose, CA): IgG1k, IgG2a, anti-CD80, anti-CD86, anti-CD3, anti-CD19, and anti–HLA-DR. The goat anti-rabbit IgG Ab conjugated to HRP. Signals were detected using the Pierce Thermo Scientific SuperSignal West Femto chemiluminescent substrate system (Thermo Scientific). Band intensities were quantified by densitometry using a Bio-Rad VersaDoc Imaging System (Bio-Rad).

Endotoxin detection

The endotoxin level in CT was determined using the Limulus assay (E-toxate; Sigma-Aldrich) following the manufacturer’s instructions. We found that 0.5 mg/ml CT, a quantity >500 times the amount used in our assays, contained ≤0.125 EU/ml endotoxin.

Chemotaxis, random migration, and chemokinesis assays

To measure chemotaxis, DCs (106 cells in 0.1 ml serum-free RPMI 1640 containing 0.5% BSA and 10 mM HEPES [pH 7.4]) were added to the upper wells of 0.33 cm2 Transwell filters (5.0-μm pore; Corning, Lowell, MA), and CXCL12 or CCL21 in a 0.6 ml volume in the same medium was transferred to the lower well. To measure random migration, 0.6 ml medium without chemokine was placed into the lower well, and for chemokinesis studies, CXCL12 was present in the medium of both the upper and lower wells. DCs were incubated in Transwells for 90 min at 37˚C under 5% CO2. Following incubation, the filters were carefully removed, and 0.5 ml medium in the lower well was collected and added to tubes containing 0.5 ml 4% paraformaldehyde in PBS. The number of migrated DCs was determined by counting cells for 1 min in a flow cytometer (LSR II, Becton Dickinson, Franklin Lakes, NJ) at a constant flow rate (~30 μl/min). In experiments in which we analyzed chemokinesis, the number of migrated DCs was determined by counting cells for 2 min. The chemotactic index represents the fold change in the number of cells that migrated in response to the chemotractant divided by the basal migration of cells that migrated in response to control medium. In the chemokinesis assays, the chemotactic index is the number of DCs that migrated in response to a gradient of CXCL12 divided by the number of DCs that migrated in symmetrical concentrations of CXCL12. Data are plotted as the mean ± SEM of triplicate measurements from two to four independent experiments. Each experiment was performed with DCs derived from different donors.

Flow cytometry

Cells were resuspended at 106 cells/0.1 ml flow buffer (PBS containing 0.5% BSA) and incubated with FITC-conjugated primary Abs for 20 min at 4˚C. Cells were then fixed with 4% paraformaldehyde for 15 min at 4˚C, washed twice with flow buffer, resuspended in 2% paraformaldehyde, and analyzed on a Becton Dickinson LSR II flow cytometer (Becton Dickinson). For indirect staining of CXCR4 and CCR7, DCs were incubated with primary Ab for 1 h at 4˚C, washed, and incubated with an FITC-conjugated secondary Ab for 30 min at 4˚C. Cells were then washed and fixed as described above. A total of 10,000 cells per sample was analyzed. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR). The geometric median fluorescence intensity (MFI) is reported. Gates showing the percentage of FITC-positive cells were set according to the isotype controls.
Endocytosis assay

Endocytosis was measured as the cellular uptake of FITC-dextran and quantified by flow cytometry. DCs (10^5 cells/sample) were incubated in complete medium containing FITC-dextran (1 mg/ml) for 30, 60, and 90 min at 4°C or 37°C. Postincubation, cells were washed three times with complete medium to remove excess dextran, and the uptake of FITC-dextran was determined by flow cytometry. A total of 10,000 cells per sample was analyzed. The MFI of the 4°C controls was <8 ± 1% (average ± SEM) of the 37°C samples (including both treated and nontreated) in three independent experiments. The MFI of the 4°C controls was subtracted from that of the 37°C samples at each time point, and the data were normalized to the MFIs of nontreated DCs (= 100%).

mRNA quantification

Individual mRNA transcripts in DCs were quantified using the NanoString nCounter gene expression system (NanoString Technologies, Seattle, WA) using an approach similar to that described by Geiss et al. (26). Briefly, 50,000 DCs per condition were lysed in RLT buffer (Qiagen, Valencia, CA) supplemented with 2-ME. Ten percent of the lysates were hybridized for 16 h with the CodeSet generated by the manufacturer and loaded into the nCounter prep station followed by quantification using the nCounter Digital Analyzer (NanoString Technologies). The nCounter data were normalized in two steps. In the first, we used the positive spiked-in controls provided by the nCounter instrument as per the manufacturer’s instructions (NanoString Technologies). Second, we normalized the data to two housekeeping genes, GAPDH and hypoxanthine phosphoribosyltransferase 1.

Quantitative phase microscopy

Immature DCs (day 3) were added to fresh complete medium and mailed overnight from The Research Institute for Children (New Orleans, LA) to Massachusetts Institute of Technology (Cambridge, MA). Upon receipt, DCs (day 4) were brought up in fresh complete medium and equilibrated at 37°C under 5% CO2 in a humidified incubator for 3 to 3 h. Then, DCs were treated with compounds and the cells incubated for an additional 3–6 h. Cells were then collected by centrifugation, washed three times in serum-free RPMI 1640 containing 10 mM HEPES (pH 7.4), plated on noncoated glass cover slips, and incubated overnight. The following day, cells were by imaged by quantitative-phase microscopy (27). Briefly, a diode laser (λ = 532 nm; Coherent, Santa Clara, CA) was used as an illumination source for an inverted microscope (IX71, Olympus, Center Valley, PA) with a 20× objective lens (0.5 numerical aperture), which provides a diffraction-limited transverse resolution of 650 nm. The additional relay optical elements were used to generate interferograms, which were measured with an EMCCD camera (Photomax 512B, Princeton Instruments, Trenton, NJ). Quantitative-phase microscopy employs the principle of laser interferometry and thus measures the full-field optical phase shift induced by the cells that can be translated to cellular thickness maps (28). The instantaneous thickness maps were measured every 1 s over 12 min, and the trajectory of center of mass for each cell was analyzed by custom Matlab Scripts (The MathWorks, Natick, MA) to retrieve the speed and persistence time. The speed and persistence time were analyzed by tracking the center of mass of each cell using techniques described previously (29), and the persistence time was calculated using the following equation (30):

\[ \langle t^2 \rangle = 2n_2 \mu \left[ t - P \left( 1 - e^{-1/P} \right) \right] + 2y, \]

in which \( n_2 \) is the number of dimensions in which the data were analyzed (= 2), \( \mu \) is the random motility coefficient, \( P \) is directional persistence time, and \( y \) is mean square positioning error.

Allogeneic MLR

DCs were treated on day 5 of culture with or without 6-Bnz-cAMP, O-Me-cAMP, or 6-Bnz-cAMP and O-Me-cAMP. After 24 h, cells were centrifuged and washed extensively in PBS and medium. DCs (50,000) were cultured with T cells (100,000/well) in a final volume of 0.2 ml in round-bottom 96-well plates for 5 d at 37°C under 5% CO2 in a humidified incubator. During the last 18 h of culture, cells were spiked with 1 μCi [3H]methyl-thymidine (20 Ci/mmol; PerkinElmer, Waltham, MA). Cells were then harvested onto UniFilter plates using a cell harvester (Perkin Elmer FilterMate Harvester, PerkinElmer). Radioactivity was counted on a PerkinElmer TopCount NXT microplate scintillation counter (PerkinElmer).

Cell viability assay

Immature DCs treated for 24 h with 6-Bnz-cAMP (100 μM), O-Me-cAMP (100 μM), a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100 μM), or LPS (1 μg/ml) were examined for viability using a Live/Dead cell viability assay (Invitrogen). None of the treatments resulted in significant cell death (the average percentage of dead cells in two independent experiments was as follows: nontreated DCs, 3.64 ± 0.33%; 6-Bnz-cAMP, 1.10 ± 0.28%; O-Me-cAMP, 1.63 ± 0.53%; 6-Bnz-cAMP and O-Me-cAMP, 1.44 ± 0.21%; and LPS, 1.34 ± 0.34%).

Statistical analysis

For the quantitative phase microscopy studies, differences between the means of experimental groups were analyzed by two-tailed Mann-Whitney rank sum test using SigmaPlot software (Systat Software, San Jose, CA). The two-tailed t test was used to analyze statistical significances between treatment groups in the CREB-phosphorylation studies, the mRNA analysis, and in studies comparing cell surface-expressed molecules by flow cytometry. Differences were considered significant when \( p < 0.05 \). In the Transwell cell migration assays, the endocytosis studies and the allogeneic MLR differences between the means of experimental groups were analyzed by single-factor ANOVA. For chemotaxis assays: NS is \( p > 0.05 \); a statistical difference between migration to medium and chemotaxis is indicated by an asterisk \(( * p < 0.05; * * p < 0.01; \) a statistical difference between migration of nontreated and treated DCs examined under the same conditions is indicated by the pound symbol \(( ^{\#} p < 0.05; ^{\#\#} p < 0.01) \). To determine the SE in the chemotactic index, we used the following equation in which Med is the number of DCs that migrated to medium (random migration), and CC is the number of DCs that migrated to chemokine:

\[ y = \frac{y_C}{M_{2M}} \left( \frac{y_C}{M_{CC}} \right)^2 + \frac{y_{2M}}{M_{2M}} \left( \frac{y_C}{M_{CC}} \right)^2. \]

Results

Selective activation of PKA, but not Epac, induces the phenotypic maturation of DCs

To investigate whether cAMP signaling via PKA is sufficient to drive the phenotypic maturation of DCs or whether cAMP activation of Epac also plays a role, we used two highly selective cAMP analogs to activate PKA or Epac (6-Bnz-cAMP or O-Me-cAMP, respectively). First, we confirmed that human monocyte-derived DCs express Epac1 by immunoblot (data not shown). These data agree with previous reports that Epac1 is expressed in human primary leukocytes, platelets, and CD34-positive hematopoietic cells (31). Next, immature DCs were treated with the PKA or Epac agonists, and expression of the costimulatory molecules CD80 and CD86 was examined by flow cytometry. We also examined the expression of CD83, a marker expressed by mature DCs (32). We found that PKA, but not Epac, increased DC expression of all three molecules (Fig. 1). To confirm these results, we showed in a separate experiment that the PKA agonist increased DC expression of costimulatory molecules and CD83 to a similar extent as that observed for DCs treated with the potent cAMP-elevating stimulus CT, the phosphodiesterase-resistant cAMP analog db-cAMP, and a phosphodiesterase inhibitor (IBMX) (Supplemental Fig. 1). These data suggest that cAMP activation of PKA, but not Epac, induces the phenotypic maturation of DCs.

To address whether crosstalk between the PKA and Epac signaling pathways regulates DC maturation, DCs were treated simultaneously with the PKA and Epac agonists. In addition to increasing the cell-surface expression of costimulatory molecules and CD83, PKA increased the expression of MHC class II (Fig. 2, compare gray histograms with bold line histograms). When the agonists were added together, Epac partially reduced the expression of CD80, CD83, CD86, and MHC class II (Fig. 2A, compare bold line histograms with thin line histograms). In contrast, the Epac agonist alone had no effect on the expression of any of these molecules (data not shown).

We next confirmed these results in 10 independent experiments using DCs derived from different donors. PKA, but not Epac,
significantly increased the cell surface expression of MHC class II, CD80, CD83, and CD86 (Supplemental Fig. 2). When the agonists were added simultaneously, Epac significantly reduced DC expression of MHC class II, CD80, and CD86. This trend was also true for CD83, although these data were not significantly different. To further validate these results, we use digital mRNA profiling to quantify transcripts encoding CD80, CD83, and CD86. DCs treated with the PKA agonist for 6 h showed a significant increase in transcripts for all three molecules relative to nontreated DCs (Fig. 2B). In contrast, the Epac agonist had no effect on transcript levels. When DCs were treated simultaneously with the PKA and Epac agonists, Epac reduced the number of transcripts for CD80 and CD83 after 6 h of incubation and significantly reduced the level of CD86 transcripts after 16 h of incubation (Fig. 2B). These data suggest that PKA–Epac crosstalk regulates the expression of CD80, CD83, and CD86 at the level of transcription. Taken together, these data demonstrate that the selective activation of PKA is sufficient to induce the phenotypic maturation of DCs and that Epac antagonizes this effect.

Crosstalk between PKA and Epac regulates DC chemotaxis to the CXCR4 ligand CXCL12

DC maturation and chemotaxis are intimately linked (33, 34). Therefore, we next tested whether PKA and Epac signaling could regulate DC chemotaxis to CXCL12. DCs were treated with the PKA or Epac agonists for 24 h and then examined for chemotaxis using Transwell cell migration assays. The PKA agonist stimulated robust chemotaxis to CXCL12 (Fig. 3A, black bars). This effect was dose-dependent and comparable to that observed for DCs treated with CT or db-cAMP (Supplemental Fig. 3A, black bars and data not shown). In contrast, the Epac agonist failed to stimulate DC chemotaxis (Fig. 3A, Supplemental Fig. 3A, black bars). The chemotactic index, a ratio of the number of DCs that migrated in response to chemokine to the number of DCs that migrated to medium, shows that both the PKA agonist and CT induced a strong chemotactic response to CXCL12 (Fig. 3B, Supplemental Fig. 3B). A novel observation was that activation of PKA induced DC migration in the absence of chemokine (random migration) (Fig. 3A, white bars, Supplemental Fig. 3A, white bars). Like the PKA agonist, both CT and db-cAMP induced random migration (Supplemental Fig. 3A, white bars and data not shown). In contrast, the Epac agonist failed to stimulate random migration. These results suggest that cAMP signaling via PKA increases the intrinsic capacity of DCs to migrate in the absence of a chemical cue.

We next examined whether crosstalk between the PKA and Epac signaling pathways regulates DC chemotaxis and random migration. DCs treated with the PKA and Epac agonists at the same time exhibited significantly reduced chemotaxis to CXCL12 when compared with DCs treated with the PKA in the presence or absence of DMSO (vehicle control for the Epac agonist) (Fig. 3A, black bars). Incubation of DCs with both agonists, however, did not affect random migration (Fig. 3A, white bars). The chemotactic index clearly demonstrates that Epac interfered with the ability of PKA to induce DC chemotaxis to CXCL12 (Fig. 3B). This set of data shows that Epac interferes with the ability of PKA to stimulate DC chemotaxis to CXCL12 but has no effect on PKA-mediated random migration.

In addition to random migration in the absence of a chemical cue, cells may exhibit nondirected migration in a uniform concentration of a chemokine, a process termed chemokinesis. We next compared the effect of PKA and Epac on DC chemokinesis in uniform concentrations of CXCL12. The PKA agonist stimulated all three forms of migration: chemokinesis, random migration, and chemotaxis (Fig. 3C). In contrast, the Epac agonist failed to stimulate any form of migration. When DCs were coincubated with the Epac and PKA agonists, Epac reduced chemotaxis to CXCL12 but failed to inhibit random migration or chemokinesis (Fig. 3C). DCs incubated with the PKA agonist in the presence of DMSO exhibited no significant differences in chemotaxis, random migration, or chemokinesis when compared with DCs treated with the PKA agonist alone, indicating that the effect of the Epac agonist on PKA-mediated chemotaxis cannot be explained by a non-specific effect of DMSO. Taken together, these data show that Epac inhibits PKA-mediated chemotaxis to CXCL12 without affecting random migration or chemokinesis.

**PKA weakly stimulates DC chemotaxis to CCL21**

We next examined the effect of cAMP signaling on DC chemotaxis to the CCR7 ligand CCL21. db-cAMP stimulated robust DC chemotaxis to CCL21 in a manner similar to that observed for LPS, a cAMP-independent stimulus known to induce migration to CCL21 (Fig. 4A, 4B). In contrast, the PKA agonist failed to stimulate chemotaxis to CCL21 but induced random migration (Fig. 4A, compare black with white bars). Treatment of DCs with a 5-fold greater concentration of the PKA agonist also failed to stimulate significant DC chemotaxis to CCL21 (data not shown). As shown for CXCL12, the Epac agonist failed to stimulate DC chemotaxis to CCL21. Furthermore, incubation of DCs with the PKA and Epac agonists simultaneously had no effect on chemotaxis. To test whether the PKA agonist could induce DC chemotaxis but with delayed kinetics, DCs were treated the PKA agonist for 48 h. At the later time point, PKA induced chemotaxis to CCL21, although the response was much less robust when compared with DCs treated with db-cAMP or LPS for 48 h (Supplemental Fig. 4). When DCs were incubated with both agonists simultaneously, chemotaxis to CCL21 was reduced when compared with DCs incubated with the PKA agonist alone. However, DMSO also reduced the ability of the PKA agonist to induce DC computed as bold line histograms. The MFIs are indicated in the upper right-hand corners of the histograms in standard type for the isotype controls and bold type for the markers. Data are representative of two independent experiments.

**FIGURE 1.** Selective activation of PKA, but not Epac, increases DC expression of costimulatory molecules. Immature DCs were treated with 6-Brz-cAMP (100 μM) or O-Me-cAMP (100 μM) for 24 h, and the surface expression of CD80, CD83 and CD86 was determined by flow cytometry. Gray histograms indicate the isotype controls, and the markers are depicted as bold line histograms. The MFIs are indicated in the upper right-hand corners of the histograms in standard type for the isotype controls and bold type for the markers. Data are representative of two independent experiments.

A novel observation was that activation of PKA induced DC migration in the absence of chemokine (random migration) (Fig. 3A, white bars, Supplemental Fig. 3A, white bars). Like the PKA agonist, both CT and db-cAMP induced random migration (Supplemental Fig. 3A, white bars and data not shown). In contrast, the Epac agonist failed to stimulate random migration. These results suggest that cAMP signaling via PKA increases the intrinsic capacity of DCs to migrate in the absence of a chemical cue.

We next examined whether crosstalk between the PKA and Epac signaling pathways regulates DC chemotaxis and random migration. DCs treated with the PKA and Epac agonists at the same time exhibited significantly reduced chemotaxis to CXCL12 when compared with DCs treated with the PKA in the presence or absence of DMSO (vehicle control for the Epac agonist) (Fig. 3A, black bars). Incubation of DCs with both agonists, however, did not affect random migration (Fig. 3A, white bars). The chemotactic index clearly demonstrates that Epac interfered with the ability of PKA to induce DC chemotaxis to CXCL12 (Fig. 3B). This set of data shows that Epac interferes with the ability of PKA to stimulate DC chemotaxis to CXCL12 but has no effect on PKA-mediated random migration.

In addition to random migration in the absence of a chemical cue, cells may exhibit nondirected migration in a uniform concentration of a chemokine, a process termed chemokinesis. We next compared the effect of PKA and Epac on DC chemokinesis in uniform concentrations of CXCL12. The PKA agonist stimulated all three forms of migration: chemokinesis, random migration, and chemotaxis (Fig. 3C). In contrast, the Epac agonist failed to stimulate any form of migration. When DCs were coincubated with the Epac and PKA agonists, Epac reduced chemotaxis to CXCL12 but failed to inhibit random migration or chemokinesis (Fig. 3C). DCs incubated with the PKA agonist in the presence of DMSO exhibited no significant differences in chemotaxis, random migration, or chemokinesis when compared with DCs treated with the PKA agonist alone, indicating that the effect of the Epac agonist on PKA-mediated chemotaxis cannot be explained by a non-specific effect of DMSO. Taken together, these data show that Epac inhibits PKA-mediated chemotaxis to CXCL12 without affecting random migration or chemokinesis.

**PKA weakly stimulates DC chemotaxis to CCL21**

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chemotaxis, suggesting that prolonged incubation with DMSO may have a nonspecific effect on DC migration. This set of data suggests that PKA signaling differentially regulates DC chemotaxis to the CXCR4 and CCR7 ligands.

PKA regulates the speed and persistence time of DC random migration

Next, we used quantitative-phase microscopy to examine the effect of PKA activation on DC random migration. Nontreated and CT-treated DCs were cultured on glass cover slips, and DC migration in medium was imaged every second over a 12-min interval. The speed (micrometers per minute) and the persistence time (time in seconds in which DCs remained on a given course before changing direction by an angle of \( \pm 60^\circ \)) were analyzed by tracking the center of mass of individual cells. CT significantly increased the speed of DC random migration over that of nontreated DCs (\( \sim 5 \mu \text{m/min versus} \sim 2.5 \mu \text{m/min}; \) Supplemental Fig. 5A, 5C, Supplemental Videos 1–4). CT treatment also increased the persistence time of DC migration compared with nontreated or LPS-treated DCs (\( \sim 7.5 \text{s versus} \sim 2.5 \text{s}; \) Supplemental Fig. 5B, 5D). Consistent with an increased speed of random migration, CT induced the dynamic formation of membrane protrusions, retractions, and membrane ruffling events in DCs that was not observed in nontreated DCs (Supplemental Videos 1–4 and data not shown). This set of data shows that CT-treated DCs migrate faster and remain on a given path for a longer duration of time before changing direction when compared with nontreated DCs.

Using the same approach, we next compared the random migration of DCs treated with the PKA agonist, CT, db-cAMP, IBMX, or LPS. Consistent with the Transwell cell migration data, the PKA agonist and CT significantly increased the speed of DC random migration when compared with nontreated or LPS-treated DCs
This was also true for DCs treated with db-cAMP or IBMX. In addition, the PKA agonist, CT, db-cAMP, and IBMX also increased the persistence time of DC random migration (Supplemental Fig. 5D). Although LPS did not affect the speed of migration, it did increase the persistence of DC migration, although less effectively than did the other compounds. Taken together, these data show that PKA signaling is sufficient to increase the speed and persistence time of DC random migration.

Crosstalk between the PKA and Epac signaling pathways regulates DC expression of CXCR4

To determine whether Epac signaling reduced DC chemotaxis to CXCL12 by downregulating CXCR4 expression, we measured DC cell surface expression of CXCR4 by flow cytometry. We found that immature DCs expressed only low levels of CXCR4 on the cell surface (Fig. 5A, thin line histogram). Consistent with the ability of PKA to induce DC chemotaxis to CXCL12, the PKA agonist increased DC expression of CXCR4 (Fig. 5A, bold line histogram). In line with our observation that Epac failed to induce DC chemotaxis to CXCL12, the Epac agonist had no effect on DC expression of CXCR4 (Fig. 5A, dotted line histogram). When DCs were incubated with the agonists simultaneously, the Epac agonist decreased CXCR4 expression (Fig. 5A, compare the bold line histogram with the gray histogram). This was also true when DCs were coincubated with the Epac agonist and CT (data not shown). None of the treatments had any effect on the staining pattern of the isotype control Ab (Fig. 5A). To confirm this result, we analyzed the effect of PKA and Epac on the expression of CXCR4 using DCs prepared from different donors (Fig. 5C). These data show that the PKA agonist significantly increased CXCR4 expression on the DC cell surface relative to nontreated DCs and that the Epac signaling had no effect on the expression of CXCR4. Epac did, however, reduce CXCR4 expression in DCs incubated with both agonists at the same time (Fig. 5C). These data show that Epac activation interferes with PKA-mediated upregulation of CXCR4 expression and are in line with the effects of these agonists on DC chemotaxis to CXCL12.

To further validate these data, we quantified RNA transcripts for the two known isoforms of CXCR4 using digital mRNA profiling. We also used this technique to examine differences in transcripts

(Supplemental Fig. 5C). This was also true for DCs treated with db-cAMP or IBMX. In addition, the PKA agonist, CT, db-cAMP, and IBMX also increased the persistence time of DC random migration (Supplemental Fig. 5D). Although LPS did not affect the speed of migration, it did increase the persistence of DC migration, although less effectively than did the other compounds. Taken together, these data show that PKA signaling is sufficient to increase the speed and persistence time of DC random migration.

Crosstalk between the PKA and Epac signaling pathways regulates DC chemotaxis to CXCL12.

A and B, Immature DCs were treated with DMSO (0.25%), 6-Bnz-cAMP (100 μM), O-Me-cAMP (100 μM), a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100 μM), or a combination of 6-Bnz-cAMP (100 μM) and DMSO (0.25%) for 24 h and examined for random migration and chemotaxis to CXCL12 (100 ng/ml). Means ± SEM of triplicate measurements from one of three independent experiments are shown in A, and the chemotactic index is displayed in B. **p < 0.01; *p < 0.05; #p < 0.05; ##p < 0.01.

C, Immature DCs were treated as described above and examined for chemotaxis to CXCL12, random migration, and chemokinesis. Means ± SEM of triplicate measurements from one of three independent experiments are shown. *p < 0.05; **p < 0.01; †p < 0.05; ††p < 0.01.

PKA–Epac crosstalk does not regulate DC chemotaxis to CCL21.

A and B, Immature DCs were treated with db-cAMP (100 μM), 6-Bnz-cAMP (100 μM), O-Me-cAMP (100 μM), a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100 μM), a combination of 6-Bnz-cAMP (100 μM) and DMSO (0.25%), or LPS (1 μg/ml) for 24 h and examined for random migration and chemotaxis to CCL21 (100 ng/ml). Means ± SEM of triplicate measurements from one of three independent experiments are shown in A, and the chemotactic index is displayed in B. **p < 0.01; *p < 0.05; ††p < 0.01.
FIGURE 5. PKA–Epac crosstalk regulates DC expression of CXCR4. A and B, Immature DCs were treated with 6-Bnz-cAMP (100 μM), O-Me-cAMP (100 μM), or a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100 μM) for 24 h, and CXCR4 expression was quantified by flow cytometry. Staining for CXCR4 is shown in A, and the IgG2a isotype control Ab is shown in B. Data are representative of three independent experiments. C, Immature DCs were treated with 6-Bnz-cAMP (100 μM), O-Me-cAMP (100 μM), a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100 μM), or a combination of 6-Bnz-cAMP (100 μM) and DMSO (0.25%) for 24 h, and CXCR4 expression was quantified by flow cytometry. The MFI (means ± SEM) from three independent experiments is shown. D–F, Immature DCs were treated with 6-Bnz-cAMP (100 μM), O-Me-cAMP (100 μM), or a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100 μM) for 6 and 16 h, after which RNA was extracted, and transcripts encoding CXCR4 isoform 1 (D) and isoform 2 (E) and CXCR7 (F) were quantified by digital miRNA profiling. The results are expressed as the mean ± SEM of duplicate measurements from three independent experiments for the 6-h data and two independent experiments for the 16-h data. *p < 0.05; **p < 0.01; #p < 0.05; ##p < 0.01.
encoding CXCR7, a newly identified second chemokine receptor for CXCL12 (35, 36). Incubation of DCs with the PKA agonist induced an increase in CXCR4 mRNA (both isoforms 1 and 2) at 6 and 16 h (Fig. 5D, 5E). In contrast, the Epac agonist had no effect on CXCR4 transcription. Importantly, Epac significantly reduced CXCR4 mRNA levels in DCs incubated with both agonists for 6 or 16 h. We also found that PKA, but not Epac, increased the abundance of CXCR7 transcripts at both time points and that Epac reduced the PKA-dependent increase in CXCR7 mRNA levels (Fig. 5F). Taken together, this set of data shows that PKA signaling is sufficient to induce CXCR4 and CXCR7 mRNA transcription and that Epac suppresses this effect.

We also examined the effect of PKA and Epac activation on DC expression of CCR7. Immature DCs expressed low levels of CCR7, and the PKA and Epac agonists added separately or together had no effect on CCR7 expression levels (Supplemental Fig. 6). These data are consistent with the inability of PKA to induce DC chemotaxis to CCL21. In contrast, LPS significantly increased CCR7 expression, and this is in line with our observation that LPS stimulated DC chemotaxis to CCL21. Treatment of DCs with the PKA agonist for a 48-h interval induced only a small increase in CCR7, and this was not affected by incubation of DCs with the Epac agonist (data not shown). These data suggest that PKA regulates DC expression of CXCR4 but has a minimal effect on expression of the CCL21 ligand CXCR7.

Epac signaling reduces PKA-mediated phosphorylation of CREB

To test the possibility that Epac signaling interferes with PKA phosphorylation of the transcription factor CREB, a known downstream target of PKA, we incubated DCs with the PKA agonist or LPS for 30 min and detected phosphorylated CREB and total CREB by immunoblot. Both the PKA agonist and LPS induced CREB phosphorylation, and the extent of phosphorylation was dependent on the dose of the PKA agonist (Fig. 6A, 6B). Next, DCs were incubated with LPS, the PKA or Epac agonists, or both agonists together, and cells were analyzed for CREB phosphorylation. Both the PKA agonist and LPS induced CREB phosphorylation, whereas the Epac agonist had no effect on CREB phosphorylation (Fig. 6C, 6D). However, Epac significantly reduced PKA-mediated phosphorylation of CREB. Data from four separate experiments show that Epac reduced the phospho-CREB signal by 20 ± 5% (average ± SEM; Fig. 6E). The CXCR4 gene promoter contains a cAMP-responsive element, and it has been shown that PKA upregulates CXCR4 expression in DCs (37). Thus, this set of data provides a molecular basis for the reduced expression of CXCR4 and impaired chemotaxis to CXCL12 observed after simultaneous activation of PKA and Epac in DCs.

**PKA–Epac crosstalk regulates DC endocytosis**

Another hallmark of mature DCs is decreased Ag uptake via endocytosis. Thus, we examined whether cAMP signaling through PKA could downregulate DC uptake of FITC-dextran over a time course of 90 min. Immature DCs actively took up dextran at each time point, and uptake was significantly reduced when DCs were induced to mature by incubation with LPS for 24 h (Fig. 7A). The PKA agonist also reduced DC uptake of dextran at each time point, whereas the Epac agonist had no significant effect on endocytosis (Fig. 7A). When DCs were incubated with the PKA and Epac agonists at the same time, Epac interfered with the ability of the PKA agonist to reduce endocytosis, and this effect could not be explained by the presence of DMSO (Fig. 7A). When data from all three time points were compiled, LPS-treated DCs took up ~61% less dextran when compared with nontreated immature DCs (Fig. 7A). The PKA agonist reduced DC uptake of dextran at each time point, whereas the Epac agonist had no significant effect on endocytosis (Fig. 7A). When DCs were incubated with the PKA agonist, endocytosis was significantly reduced when DCs were induced to mature by incubation with LPS for 24 h (Fig. 7A). The PKA agonist also reduced DC uptake of dextran at each time point, whereas the Epac agonist had no significant effect on endocytosis (Fig. 7A).
PKA–Epac crosstalk regulates DC maturation

The PKA agonist also induced DC maturation as evidenced by a ∼50% decrease in dextran uptake when compared with immature DCs. Finally, treatment of DCs with both agonists reduced endocytosis by ∼26%, whereas treatment with the Epac agonist alone only slightly reduced endocytosis (∼11%).

PKA–Epac crosstalk regulates DC cytokine production

To further examine PKA–Epac regulation of DC function, we used mRNA profiling to quantify the effect of PKA and Epac signaling on cytokine production. When compared with nontreated immature DCs, DCs treated with the PKA agonist exhibited reduced levels of mRNA transcripts encoding TNF-α, TGF-β, IL-18, and IL-10 (Fig. 8A–G). The Epac agonist alone had no effect on the level of transcripts encoding these cytokines. When DCs were incubated with both agonists, Epac had no effect on TNF-α and TGF-β mRNA levels, but partially reversed the inhibitory effect of PKA on IL-18 and IL-10 mRNA levels following 6 h of incubation. None of the treatments had any effect on the already low levels of IL-12 mRNA in immature DCs (data not shown). Taken together, these data suggest that PKA suppresses cytokine production by DCs and that Epac partially reverses this effect.

Discussion

The cAMP-dependent signaling pathways that regulate DC maturation remain to be completely defined. This led us to investigate whether cAMP signaling via PKA is sufficient to drive maturation...
or whether cAMP activation of Epac is also involved. To address this question, we used cAMP analogs specific for PKA or Epac to discriminate the effect of these signaling pathways on the maturation of human monocyte-derived DCs. The experiments presented in this study show that selective activation of PKA stimulates DC maturation. Epac signaling has no effect on maturation. However, when both pathways are activated simultaneously, Epac antagonizes the effect of PKA on the phenotypic maturation and function of DCs. Specifically, we found that the cell surface expression of MHC class II, costimulatory molecules, the maturation marker CD83, and the chemokine receptor CXCR4 was reduced when DCs were treated with the PKA and Epac agonists at the same time. Epac also interfered with PKA activation of CREB, suggesting that PKA–Epac crossstalk may converge at the level of transcription to regulate DC maturation. Further, Epac antagonized the effect of PKA on DC function. Epac partially reversed the effect of PKA on endocytosis, chemotaxis to CXCL12, cytokine production, and T cell activation. These findings suggest that the net effect of cAMP signaling is not simply dictated by the action of PKA or Epac alone, but involves a complex integration of the two signaling pathways.

A novel finding of this study was that in addition to upregulating CXCR4, PKA activation increased DC transcription of CXCR7. Whereas CXCR4 and CXCL12 were once thought to form a monogamous pair, CXCR7 was recently identified as a second chemokine receptor for CXCL12 (35, 36, 38–44). Unlike other chemokine receptors, CXCR7 does not stimulate chemotaxis (36). Rather, CXCR7 is thought to belong to the atypical chemokine receptor family that plays a role in scavenging or altering the localization of chemokines via binding and/or internalizing the chemokines without inducing signal transduction (45). A prevailing theory is that CXCR7 may function to scavenge CXCL12 at the trailing edge of migrating cells, thus creating a local chemokine gradient around the cell to maintain the directionality of migration (46–50). Interestingly, we also found that PKA increased the transcription of two isoforms of CXCR4 and that Epac partially antagonized this effect. The significance of PKA-mediated up-regulation of both CXCR4 isoforms and CXCR7 for DC chemotaxis to CXCL12 remains to be elucidated.

An unexpected finding of this study was that DCs treated with the PKA agonist migrated strongly to CXCL12 but poorly to CCL21. In humans, it is currently not known whether CCR7 is the dominant chemokine receptor for directing DC migration to lymph nodes or whether CXCR4 also plays a role. In murine DCs, both CCR7 and CXCR4 direct migration to lymphoid tissues (38, 41, 42, 51–59). CCR7-deficient mice and plt/plt mutant mice (which lack both CCR7 ligands CCL19 and CCL21) exhibit a severe defect in DC migration from the skin to the draining lymph nodes. The observed defect is still incomplete, suggesting the involvement of another chemokine receptor. Indeed, CXCR4 is required for the migration of murine DCs (both Langerhans cells and dermal DCs) to the skin-draining lymph nodes (60). In humans, CXCR4 is required for the chemotaxis of DCs from the epidermis to the dermis (54). CXCR4 also functions synergistically with CCR3 to induce the migration of human plasmacytoid DCs to lymph nodes (61). In addition to regulating chemotaxis, CXCL12 promotes murine DC survival and maturation (62). Our preliminary results show that CXCL12 increases human DC expression of MHC class II and costimulatory molecules (J. Garay and B.L. Dickinson, unpublished observation). Thus, PKA-dependent upregulation of CXCR4 may augment both DC maturation and homing to lymphoid tissues.

DCs exhibit nondirectional migration in the absence of chemical cues (random migration) and in symmetrical concentrations of chemoattractants (chemokinesis). We found that PKA activation induced both forms of migration. It is thought that random migration may function to prime DC chemotaxis by sensitizing the chemotaxis machinery (63, 64). For example, PKA signaling has been shown to lower the threshold for chemokine receptor detection of chemokines and facilitate cell migration in response to distant or suboptimal chemokine signals (65). Another role for nondirectional migration may be to increase the frequency of DC contacts with T cells within the crowded confines of lymphoid tissues, where chemokine gradients may play a less important role in directing cell migration (66, 67). Our data show that although both the PKA agonist and CT induced DC random migration, only the PKA agonist stimulated chemokinesis. One possibility to explain this result is that the PKA agonist may activate PKA more potently than CT. We also found that Epac signaling failed to stimulate DC random migration and chemokinesis. And, although Epac partially inhibited the chemotaxis of PKA-treated DCs, Epac had no effect on the ability of PKA to induce DC random migration or chemokinesis. These results suggest that the cAMP-dependent mechanisms that direct chemotaxis, random migration, and chemokinesis may be differentially regulated in DCs.

In most cases, the ultimate consequence of DC maturation for the immune response is T cell activation. T cell differentiation into Th1, Th2, Th17, or T regulatory effectors is strongly influenced by the pattern of cytokines released by DCs during Ag presentation. We found that transcription of TNF-α, TGF-β, IL-18, and IL-10 was suppressed in DCs treated with the PKA agonist. This result is in line with the observation that cAMP-elevating bacterial toxins, such as CT, the E. coli heat-labile enterotoxin, pertussis toxin, and adenylate cyclase toxin activate human monocyte-derived DCs and inhibit cytokine production (68, 69). CT and heat-labile enterotoxin are potent mucosal adjuvants that act on DCs to promote T cell responses to codelivered protein Ags (18, 70–74). Despite the inhibitory effect of PKA on cytokine expression, we found that PKA-treated DCs stimulated allogeneic T cell proliferation in vitro. Although Epac had no effect on cytokine transcript levels,

![Figure 9](http://www.jimmunol.org)
it partially reversed the inhibitory effect of PKA on the transcription of IL-18 and IL-10. Epac also diminished the capacity of PKA-activated DCs to stimulate T cell proliferation. Thus, Epac may function as a molecular brake to attenuate the magnitude of PKA signaling to prevent excessive T cell activation. Our data suggest that PKA–Epac crosstalk may converge at the level of transcription to regulate DC maturation and function. In support of this idea, we show that Epac interferes with PKA phosphorylation of CREB, and others have shown that Epac regulates the activity of the C/EBP family of transcription factors (75, 76). How cAMP signaling regulates transcription may depend on the strength, duration, and timing of PKA and Epac activation. These signals also must be integrated with signaling events initiated by DC interaction with pathogen-associated molecules, such as TLR ligands, as well as with signaling molecules that originate from neighboring cells (77).

In summary, the results of this study have important implications for DC-based cancer vaccines. A current focus is on the development of novel stimuli for the ex vivo conditioning of DCs to promote their maturation and migration to lymph nodes for T cell priming. Future studies will be required to determine whether stimuli that activate PKA generate mature, immunogenic DCs capable of activating T cells in vivo and, conversely, whether stimuli that target Epac generate tolerogenic DCs capable of attenuating allergic and autoimmune disease (6, 78).

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Disclosures

The authors have no financial conflicts of interest.

References


B. Homey, C. Massacrier, B. Vanbervliet, A. Zlotnik, and A. Vicari. 2000. Regulation of dendritic cell re-

32. Pacold, E., H. Hengartner, and B. Ludewig. 2002. Antiviral immune responses in the ab-

33. Dickinson, B. L., and J. D. Clements. 1995. Dissociation of Escherichia coli

cAMP-dependent induction of the SOCS-3 gene by cyclic AMP in COS1 cells.


NF-κB by the Gram-negative bacterium Borrelia burgdorferi.


Cutaneous dendritic cell responsiveness to the constitutive chemokine stromal


39. Wei, S. H., I. Parker, M. J. Miller, and M. D. Cahan. 2003. A stochastic view of

lymphocyte motility and trafficking within the lymph node. Immunol. Rev.

195: 136–159.


repertoire scanning is promoted by dynamic dendritic cell behavior and random


2002. Cholera toxin and heat-labile enterotoxin activate human monocyte-
derived dendritic cells and dominantly inhibit cytokine production through a cy-


2002. Pertussis toxin and the adenylyl cyclase toxin from Bordetella pertussis

activate human monocyte-derived dendritic cells and dominantly inhibit cyto-

kine production through a cAMP-dependent pathway. J. Leukoc. Biol. 72: 962–

969.

43. Anosova, N. G., S. Chabot, V. Shrechedar, J. A. Borawski, B. L. Dickinson, and


derivatives induce dendritic cell migration into the follicle-associated epithelium


47. Dickinson, B. L., and J. D. Clements. 1995. Dissociation of Escherichia coli

heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. In-


CC/CAAT/enhancer-binding proteins as protein activated by cAMP-

activated transcription factors that mediate the induction of the SOCS-3 gene. J.


49. Borland, G., R. J. Bird, T. M. Palmer, and S. J. Yuswod. 2009. Activation of protein kinase C alpha by EPC1 is required for the ERK- and EBPeta-

dependent induction of the SOCS-3 gene by cyclic AMP in COS1 cells. J. Biol.

Chem. 284: 17391–17403.


51. Xiao, B. G., Y. M. Huang, and H. Link. 2003. Dendritic cell vaccine design:

strategies for eliciting peripheral tolerance as therapy of autoimmune diseases.

BioDrugs 17: 103–111.

52. Kabashima, K., N. Shiraishi, K. Sugita, T. Mori, A. Ounoe, M. Kubayashi, J.


engagement is required for migration of cutaneous dendritic cells. Am. J. Pathol.


53. Vanbervliet, B., N. Bendriss-Vermare, C. Massacrier, B. Homey, O. de Bouteiller, F.

Briere, G. Trinchieri, and C. Caux. 2003. The inducible CXCR3 ligand controls plasma-myeloid dendritic cell responsiveness to the constitutive chemokine stromal


54. Ouwehand, K., S. J. Santegoets, D. P. Bruynzeel, R. J. Scheper, T. D. de Gruijl, and

M. C. Dieu-Nosjean And, and A. Vicari. 2002. Regulation of dendritic cell re-

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Legends for Supplemental Figures 1-6 and Movies 1-4

Supplemental figure 1. Cyclic AMP signaling and activation of PKA increases dendritic cell expression of co-stimulatory molecules. Immature DCs were treated with 6-Bnz-cAMP (100μM), CT (1μg/ml), db-cAMP (100μM) or IBMX (1mM) for 24 h and the surface expression of CD80, CD83 and CD86 was determined by flow cytometry. Grey histograms indicate the isotype controls and the markers are depicted in the bold line histograms. The MFI s are indicated in the upper right hand corners of the histograms in standard type for the isotype controls and bold type for the markers. Data are representative of three independent experiments for CT, 6-Bnz-cAMP and IBMX, and two independent experiments for db-cAMP.

Supplemental figure 2. PKA-Epac crosstalk regulates dendritic cell expression of MHC class II and co-stimulatory molecules. A-D, Immature DCs were treated without or with 6-Bnz-cAMP (100μM), O-Me-cAMP (100μM) or a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100μM) for 24 h and the surface expression of CD80, CD83, CD86 and MHC class II was determined by flow cytometry. The MFI s (means ± SEM) from ten independent experiments are plotted. Statistical significance between non-treated DCs and treated DCs (#), and between PKA agonist-treated DCs and DCs treated with the PKA and Epac agonists in combination (*) is indicated.

Supplemental figure 3. Selective activation of PKA induces DC random migration, chemokinesis and chemotaxis to CXCL12. A and B, Immature DCs were treated with CT (1μg/ml), 6-Bnz-cAMP (100μM or 500μM) or O-Me-cAMP (100μM or 500μM) for 24 h and
examined for random migration and chemotaxis to CXCL12 (100ng/ml). Means ± SEM of triplicate measurements from one of two independent experiments for O-Me-cAMP, and one of three independent experiments for CT and 6-Bnz-cAMP are shown in panel A and the chemotactic index is displayed in panel B.

**Supplemental figure 4.** PKA-Epac crosstalk does not regulate DC chemotaxis to CCL21. Immature DCs were treated with db-cAMP (100μM), 6-Bnz-cAMP (100μM), O-Me-cAMP (100μM), a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100μM), a combination of 6-Bnz-cAMP (100μM) and DMSO (0.25%) or LPS (1μg/ml) for 48 h and examined for random migration and chemotaxis to CCL21 (100ng/ml). Means ± SEM of triplicate measurements from one experiment is shown.

**Supplemental figure 5.** Selective activation of PKA increases the speed and persistence time of dendritic cell random migration. A and B, DCs were treated without (no treatment) or with CT (1μg/ml) for 24 h and examined for random migration by quantitative phase microscopy. The speed (A) and persistence time (B) of migration are shown. Results are from one experiment in which 38 DCs were examined per condition. C and D, DCs were treated without (no treatment) or with CT (1μg/ml), db-cAMP (100μM), 6-Bnz-cAMP (100μM), IBMX (1mM) or LPS (1μg/ml) for 24 h and examined for random migration by quantitative phase microscopy. The speed (C) and persistence time (D) of migration are shown. Results are from one experiment in which 75 DCs were examined per condition.
**Supplemental figure 6.** PKA-Epac crosstalk does not regulate dendritic cell expression of CCR7. Immature DCs were treated with or without 6-Bnz-cAMP (100μM), O-Me-cAMP (100μM), a combination of 6-Bnz-cAMP and O-Me-cAMP (both 100μM), a combination of 6-Bnz-cAMP (100μM) and DMSO, or LPS (1μg/ml) for 24 h and CCR7 expression was examined by flow cytometry. Grey histograms indicate the isotype controls and the markers are depicted by bold line histograms. The MFI s are indicated in the upper right hand corners of the histograms in standard type for the isotype controls and bold type for the markers. Data are representative of 2 independent experiments.

**Movies 1-4.** Cholera toxin induces dendritic cell random migration. Immature DCs were treated without (Movies 1-2) or with CT (1μg/ml) (Movies 3-4) for 24 h and then imaged for random migration using quantitative phase microscopy as described in the *Materials and Methods.*
Supplemental figure 1

CD80

No treatment

PKA agonist

6-Bnz (100 M)

CT (1 M)

db-c-AMP (100 M)

IBMX (1 mM)

No treatment

98 86.0

132 89.5

117 89.2

117 90.2

114 75.1

CD83

98 200

132 55.3

117 91.1

117 92.5

114 90.7

CD86

98 90.4

132 95.8

117 96.4

117 97.0

114 94.3

Supplemental figure 1
Supplemental figure 2

A  MHC II

B  CD80

C  CD83

D  CD86
Supplemental figure 3

**A**

- No treatment
- CT 1 µg/ml
- PKA agonist (6-Bnz 100 µM, 500 µM)
- Epac agonist (6-Bnz 100 µM, O-Me 100 µM, O-Me 500 µM)

**B**

- No treatment
- CT 1 µg/ml
- 6-Bnz 100 µM, 500 µM
- O-Me 100 µM, 500 µM

Number of migrated cells vs. Chemotactic index
Supplemental figure 4
Supplemental figure 5
Supplemental figure 6

No treatment

6-Bnz-cAMP + O-Me-cAMP

6-Bnz-cAMP (100uM)

6-Bnz-cAMP + DMSO

O-Me-cAMP (100uM)

LPS 1ug/ml

Cell number

Log fluorescence intensity

Supplemental figure 6