Phosphodiesterase 4 inhibitors reduce human dendritic cell inflammatory cytokine production and Th1-polarizing capacity

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Abstract

Inhibitors of cAMP-specific phosphodiesterase (PDE) 4 have been shown to inhibit inflammatory mediator release and T cell proliferation, and are considered candidate therapies for Th1-mediated diseases. However, little is known about how PDE4 inhibitors influence dendritic cells (DC), the cells responsible for the priming of naive Th cells. Therefore, we investigated the PDE profile of monocyte-derived DC, and whether PDE4 inhibitors modulate DC cytokine production and T cell-polarizing capacity. We mainly found cAMP-specific PDE4 enzymatic activity in both immature and mature DC. In contrast to monocytes that mainly express PDE4B, we found that PDE4A is the predominant PDE4 subtype present in DC. Immature DC showed reduced ability to produce IL-12p70 and tumor necrosis factor (TNF)-α upon lipopolysaccharide or CD40 ligand (CD40L) stimulation in the presence of PDE4 inhibitors, whereas cytokine production upon CD40L stimulation of fully mature DC in the presence of PDE4 inhibitors was not affected. Exposure to PDE4 inhibitors for 2 days during DC maturation did not influence T cell-stimulatory capacity or acquisition of a mature phenotype, but increased the expression of the chemokine receptor CXCR4. Furthermore, DC matured in the presence of PDE4 inhibitors showed reduced capacity to produce IL-12p70 and TNF-α upon subsequent CD40L stimulation. Using these PDE4 inhibitor-matured DC to stimulate naive T cells resulted in a reduction of IFN-γ-producing (Th1) cells. These findings indicate that PDE4 inhibitors can affect T cell responses by acting at the DC level and may increase our understanding of the therapeutic implication of PDE4 inhibitors for Th1-mediated disorders.

Introduction

In the immune system, cAMP signaling pathways are important for the control of a wide range of cellular functions, including mediator release and proliferation. The level of the second messenger cAMP is tightly regulated at the level of synthesis (1) and hydrolysis. Phosphodiesterases (PDE) are the critical enzymes responsible for the degradation of cAMP and/or cGMP. Based on genetic, biochemical and pharmacological data, PDE enzymes have been classified into 11 distinct families (2,3). Immune and inflammatory cells predominantly express PDE4 followed by PDE3 and, to a lesser extent, PDE7 family members (3,4). The PDE4 family, the main contributor to cAMP hydrolysis, is encoded by four distinct genes (PDE4A, B, C and D) and comprises >15 PDE4 isoforms.

Since an increase in cAMP has been shown to inhibit inflammatory and immunological processes, the PDE families have been proposed as targets for therapeutic intervention in pathologies such as allergies and autoimmune diseases (5). PDE4-specific inhibitors, such as Rolipram and Cilomilast (6), have been demonstrated to elevate cAMP levels, and inhibit proliferation, cytokine production and mediator release of several cells, including T cells, monocytes and eosinophils [reviewed in (4,7)]. However, little is known about how PDE4 inhibitors influence the inflammatory response or initiation of the specific immune response at the level of dendritic cells (DC).

DC are professional antigen-presenting cells (APC) that play a crucial role in the primary immune response because of...
their unique ability to activate naive Th cells (ThN) (8). Immature DC in the peripheral tissues efficiently capture and process invading pathogens, and this induces their activation and migration to the draining lymph nodes. DC activation and maturation can be initiated by several factors, such as bacteria or their products [e.g. lipopolysaccharide (LPS)], tissue-derived inflammatory cytokines [e.g. IL-1β] and tumor necrosis factor (TNF-α) or the interaction between CD40 on the DC and CD40 ligand (CD40L) on activated Th cells (9–11). In addition, LPS and CD40L can trigger secretion of IL-12 and other inflammatory cytokines such as TNF-α by DC (10, 12). IL-12 plays a central role in the induction of IFN-γ production by Th cells, enhances NK cell cytotoxicity, and promotes the development of cytotoxic T cells [reviewed in (13)].

Previous studies have shown that modulation of DC-derived IL-12 strongly influences the outcome of the immune response. Pharmacological agents such as β-agonists (14) and glucocorticoids (15) can modulate inflammatory cytokine production and immune activation of T cells via their direct effect on DC. In the present study, we characterized the PDE profile of DC generated in vitro from monocytes. In addition, we examined whether the PDE4 inhibitors Rolipram and Ciclopiroxin influenced DC phenotype and immune functions such as cytokine production, stimulation of T cell proliferation and the capacity of these DC to polarize Th,N. We show predominant cAMP-hydrolyzing activity of PDE4 in DC. PDE4-specific inhibitors reduced IL-12p70 and TNF-α production, but did not influence the T cell-stimulator capacity of DC. Moreover, PDE4 inhibitor-treated DC showed reduced capacity to stimulate IFN-γ production by activated Th,N. This indicates that PDE4 inhibitors can modulate the outcome of T cell responses via their anti-inflammatory action on DC.

Methods

Cytokines and reagents

The PDE inhibitors Rolipram [4-(3-cyclopentoxy-4-methoxyphenyl)-2-(1H)-pyrrolidone], Ciclopiroxin (Ariflo™), SB207499 [c-4-cyano-4-(3-cyclopentoxy-4-methoxyphenyl)-r-1-cyclohexanecarboxylic acid], SKF 95654 (4,5-dihydro-6-[4-(1,4-dihydro-4-oxopyridin-1-yl)phenyl][5-methyl-3(2H) pyrazidinone] and CI-930 (4,5-dihydro-6-[4-(1H)imidazol-1-yl)phenyl][5-methyl-3(2H) pyrazidinone] were synthesized by Pfizer GRD (Fresnes Laboratories, Chemistry Department, Fresnes, Switzerland) by density centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) according to the supplier’s instructions. Subsequently, PBMC were treated according to the protocol recommended by Miltenyi Biotec and the resulting monocyte fraction (95–98% CD14+) was then processed for in vitro culture in RPMI 1640 (Invitrogen Life Technologies, Cergy Pontoise, France) containing 10% FCS, 1% non-essential amino acids, 1% sodium pyruvate, 100 µg/ml kanamycin (all from Invitrogen Life Technologies) and supplemented with rhGM-CSF (500 U/ml) and rhIL-4 (250 U/ml). At day 3, the medium including the supplements was refreshed. In some experiments, monocyte-derived DC were prepared by Percoll gradient centrifugation followed by culture of adherent cells as previously described (15). In both protocols, similar preparations of immature CD14+CD14- DC were obtained at day 6 as evaluated by phenotypic analysis.

Induction of maturation of DC in the presence or absence of PDE4 inhibitors

At day 6, the maturation of CD1a+ DC was induced by a 2-day exposure to either LPS alone (250 ng/ml) or a combination of the cytokines rhIL-1β (10 ng/ml) and rhTNF-α (25 ng/ml). For phenotypic analysis and mixed lymphocyte reaction experiments, DC maturation was induced by LPS (1 µg/ml) in the presence of IFN-γ (10imon/ml). In addition, maturation was induced in the absence (vehicle DMSO) or presence of PDE4 inhibitors (10 µM of Rolipram or Ciclopiroxin, unless stated otherwise).

Induction of cytokine secretion by DC

Immature DC (at day 6) or mature DC (day 8) were harvested, washed extensively and 2 × 10⁶ cells/well were stimulated with CD40L-transfected J558 cell line (2 x 10⁶ cells/well) in 96-well flat-bottom culture plates (Costar, Cambridge, MA). J558 myeloma cells transfected with CD40L and the mock-transfected J558 were a generous gift of Dr E. Padovan (Basel, Switzerland). In some experiments, cytokine production was also evaluated after stimulating immature DC with LPS (1 µg/ml) and IFN-γ (10³ U/ml). Supernatants were harvested after 24 h and stored at –20°C until determination of IL-12p70 and TNF-α levels by ELISA. Measurement of IL-12p70 and TNF-α levels in culture supernatants were performed using OptEIA human IL-12p70 and TNF-α antibody sets (BD Biosciences Pharmingen, Le Pont de Claix, France). The detection limit for both IL-12p70 and TNF-α is 10 pg/ml.
Analysis of expression of cell-surface molecules by flow cytometry

Mouse mAb against the following human molecules were used: CD4 (OKT4; Ortho Diagnostic Systems, Beerse, Belgium), CD45RO (UCHL-1; a gift from Dr P. Beverley, London, UK). CD1a-phycocythrin (PE) (SFC197/1y1AB), CD14-FITC (RM502), CD83-FITC (HB15a), mannose receptor (MR)-PE (3.29B1.10), CD45RA-PE (L307.4), CD86-PE (FUN-1), HLA-DR-PE (MOPC-195) were all obtained from Beckman Coulter (Fullerton, CA). CD3-PE (UCHT1), CD40-FITC (5C3), CD80-PE (L307.4), CD86-FITC (FUN-1), HLA-DR-FITC (L243) and CXCR4-PE (12G5) were obtained from BD Biosciences Pharmingen.

Mixed lymphocyte reactions

At day 6, immature DC were induced to mature for 48 h with either LPS and IFN-γ or IL-1α and TNF-α in the presence of PDE4 inhibitors or vehicle DMSO, and were then extensively washed. Graded doses of differently matured DC (up to 10⁴ cells/well) were plated in 96-well round-bottom culture plates with 10,000 DC/well in the presence of 1 ng/ml staphylococcal enterotoxin B (SEB; Sigma). After 5 days of co-culture, IL-2 (10 U/ml; Cetus, Emeryville, CA) was added and the cultures were further expanded for another 9 days. On day 14, resting memory T cells were harvested, washed, and re-stimulated for 6 h with 10 ng/ml phorbol myristate acetate, 1 μg/ml ionomycin and 10 μg/ml Brefeldin A (all obtained from Sigma). Cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with PBS containing BSA (1%) and saponin (0.5%), stained with FITC-labeled anti-IFN-γ and PE-labeled anti-IL-4 mAb (BD Biosciences Pharmingen), and analyzed on a FACScan (BD Biosciences Pharmingen).

PDE enzymatic activity assay

Cells were homogenized and sonicated, and PDE activities were assayed using a modification of the method described previously (16). Briefly, 10 μl of cell lysate supernatant and either DMSO or 3 mM of the respective PDE1, PDE3 and PDE4 inhibitors [vinpocetine (17), SKF 95654 (18) and Rolipram] was diluted in a 96-well plate to a final volume of 200 μl containing the assay buffer (40 mM Tris–HCl, pH 8, 0.5 mM MgCl₂, 4 mM β-mercaptoethanol, 10 nM [³H]cAMP and 1 μM cAMP). After 1 h, the reaction was stopped by the addition of 1 μM [¹⁴C]cAMP in TFA (0.5%). Membrane-bottom microtiter plates (Silent Monitor; Nalgene Nunc, Rotherwas, Hereford, UK) filled with 50 μg alumina were equilibrated with 0.1 M TES–NaOH, pH 8.0. The reaction mixtures were then applied to the columns. The non-hydrolyzed cAMP was eluted with 3 ml equilibration buffer. The [³H]cAMP and [¹⁴C]cAMP were then eluted with 2 mM NaOH directly into scintillation vials containing alkali-compatible scintillant (Ultima Gold; Perkin Elmer Life Sciences, Zaventem, Belgium). The separated [³H]cAMP was background-corrected for recovery using the separated and non-separated [¹⁴C] values, and expressed as a fraction of the total [³H]cAMP to give the amount of the substrate hydrolyzed. The activity of each isoenzyme was defined as the difference in activity with/without PDE inhibitor.

RT-PCR amplification

Total RNA was purified using the RNeasy Midi kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. cDNA was generated from 1 μg total RNA using the First Strand cDNA synthesis kit for RT-PCR (AMV) from Roche Molecular Biochemicals (Meylan, France), and directly amplified by PCR after the addition of specific primers and Taq DNA polymerase (Roche Molecular Biochemicals). Oligonucleotide primers were as follows: PDE4A (L20965), 5’-TCGGAGCTGCGCTCATGTA-3’ and 5’-GGCACTGTTGCTGTCACTAT-3’; PDE4B (L20966), 5’-AGCTCATAGCCATAAGTG-3’ and 5’-GAGCGTGTCAGGCTGTTGTA-3’; PDE4C (L20967), 5’-CCCTGCTGACTGCTGGAGG-3’ and 5’-CAGCAGAATCTGGAGG-3’; PDE4D (L20970), 5’-GGCTCCAACAGTAAAA-3’ and 5’-CCAGACAACTCTGACTTT-3’. All the PDE subtype PCR fragments corresponded to conserved regions derived from the gene of each PDE subtype and the primers were able to detect all the members of the same subtype family. The number of PCR cycles was optimized to be in the exponential phase of the reaction and a constitutive marker, human G3PDH, was included using a commercially available primer set defining a 452-bp product (Clontech, Palo Alto, CA). For each RNA sample, controls lacking reverse transcriptase were included in the PCR reactions. Reactions were performed with an initial holding step at 94°C for 120 s, followed by 94°C for 60 s, 60°C for 120 s, 72°C for 120 s during 32 cycles and a final holding step at 72°C for 5 min. PCR products along with mol. wt markers (SmartLadder SF; Eurogentec, Angers, France) were analyzed on a 1.4% agarose gel containing ethidium bromide.

Isolation of CD4⁺CD45RA⁺CD45RO⁻ naive Tₙ cells, co-cultures with DC and induction of memory-type cytokines in Tₙ cells

Tₙ cells were isolated from peripheral blood leukocytes with the negative selection human CD4⁺/CD45RO⁻ column kit (R & D Systems). This method yielded highly purified (>98%) CD4⁺CD45RA⁺CD45RO⁻ T cells as assessed by flow cytometry. DC that had been matured for 2 days with rhIL-1α and TNF-α, either in the absence or presence of PDE4 inhibitors, were extensively washed to remove any residual factors. Tₙ cells (20,000 cells/well) were co-cultured in 96-well flat-bottom culture plates with 10,000 DC/well in the presence of 1 ng/ml staphylococcal enterotoxin B (SEB; Sigma). After 5 days of co-culture, IL-2 (10 U/ml; Cetus, Emeryville, CA) was added and the cultures were further expanded for another 9 days. On day 14, resting memory T cells were harvested, washed, and re-stimulated for 6 h with 10 ng/ml phorbol myristate acetate, 1 μg/ml ionomycin and 10 μg/ml Brefeldin A (all obtained from Sigma). Cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with PBS containing BSA (1%) and saponin (0.5%), stained with FITC-labeled anti-IFN-γ and PE-labeled anti-IL-4 mAb (BD Biosciences Pharmingen), and analyzed on a FACScan (BD Biosciences Pharmingen).
Results

Comparison of PDE profiles of monocyte-derived DC and monocytes

The total cAMP-hydrolyzing activity of different cellular preparations was measured in the absence or presence of PDE1, PDE3 and PDE4 inhibitors to compare the PDE activity profile of both monocytes and monocyte-derived DC from the same donor. Monocytes were either frozen for later PDE activity determination or cultured with GM-CSF and IL-4 to generate DC. Immature DC were either frozen at day 6 for PDE activity measurement or induced to mature for 24 h with LPS or LPS and IFN-γ. PDE activity was determined in parallel in all cell types and the results obtained are shown in Fig. 1. We found that PDE4 represents the major cAMP-hydrolyzing activity in monocytes, immature DC and mature DC. PDE4 activity, as estimated using the PDE4-specific inhibitor Rolipram, accounted for up to 60% of the total cAMP-hydrolyzing activity and this proportion was not grossly modified following activation of the DC. In addition, as identified by the use of specific inhibitors, enzymatic activities for PDE1 and PDE3 were found in monocytes, and to a somewhat lesser extent in DC. It is interesting to note that DC showed increased activity of a non-PDE1,-PDE3,-PDE4 isoenzyme when compared with monocytes.

To further examine the nature of the PDE4 activity, mRNA expression of the different PDE4 subtypes (PDE4A, B, C and D) was determined in monocytes and immature DC. RT-PCR was performed in parallel on cellular preparations obtained from four or five human donors. As depicted in Fig. 2, PDE4B is the major PDE4 subtype found in resting monocytes, followed by PDE4A and PDE4D, whereas PDE4C could not be detected. Interestingly, a different expression pattern of the PDE4 subtypes was observed in DC compared to monocytes (Fig. 2). PDE4A was the most abundant subtype found in DC, while PDE4B and PDE4D were less expressed, and PDE4C was not detectable at all.

Inhibitory effect of PDE4 inhibitors on cytokine secretion by immature DC

Since we found that PDE4 was the main PDE enzymatic activity present in DC, we studied whether PDE4-specific inhibitors could affect cytokine production by immature DC. Immature DC were incubated with graded doses of PDE4 inhibitor Rolipram with the cAMP analogue db-cAMP, which is able to block all cAMP-specific PDE families, on IL-12p70 and TNF-α production by immature DC. As shown in Fig. 3, stimulation of immature DC with LPS and IFN-γ in the presence of increasing concentrations of Rolipram resulted in a dose-dependent inhibition of IL-12p70 (closed circles) concentration of triplicate cultures in 24 h supernatants from one representative experiment of four. SD < 10% (not shown).
PDE4 inhibitors reduce T_h1 responses by affecting DC-derived IL-12

<table>
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<th>Compounds</th>
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<td>LPS + IFN-γ</td>
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<td>7</td>
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<tr>
<td>Cilomilast</td>
<td>LPS + IFN-γ</td>
<td>46 ± 23</td>
<td>5</td>
</tr>
<tr>
<td>db-cAMP</td>
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<tr>
<td>Cilomilast</td>
<td>J558-CD40L</td>
<td>70 ± 23</td>
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Table 1. PDE4 inhibitors reduce inflammatory cytokine production by immature DC

10 μM PDE4 inhibitors. Therefore, we used this dose for further experiments. As depicted in Table 1, the PDE4-specific inhibitor Cilomilast showed a similar inhibitory effect on DC cytokine secretion when compared with Rolipram. The down-regulation of IL-12p70 production by PDE4 inhibitors was more pronounced when DC were stimulated with CD40L-transfected cells (inhibition 68–70%) instead of LPS and IFN-γ (inhibition 37–46%). The inhibition of TNF-α production by both PDE4 inhibitors ranged between 48 and 62%, and was comparable using both modes of activation.

Presence of PDE4 inhibitors during maturation of DC does not affect the acquisition of a mature phenotype, but enhances CXCR4 expression

We studied whether the presence of PDE4 inhibitors during DC maturation could affect the expression of markers associated with DC maturation. Immature DC were induced to mature with LPS and IFN-γ either in the absence or presence of the PDE4 inhibitors Rolipram or Cilomilast. The exposure of DC to PDE4 inhibitors did not influence the up-regulation of surface expression of CD83, HLA-DR, CD40, CD80 and CD86 respectively, neither was down-regulation of MR expression affected when compared with vehicle-treated control cells (Fig. 4). However, PDE4 inhibitors induced up-regulation of cell-surface expression of CXCR4 on DC when compared with control cells. Similar results were obtained when TNF-α and IL-1β were used to induce DC maturation (data not shown), suggesting that the effect of PDE4 inhibitors on DC does not critically depend on the activation condition in the microenvironment.

PDE4 inhibitors do not influence T cell stimulatory capacity of DC when present during DC maturation

Next, we questioned whether the ability of DC to activate the proliferative response of T cells was modified by PDE4 inhibitors. DC were induced to mature either by LPS and IFN-γ or a combination of the maturation factors (MF) IL-1β and TNF-α in the presence of PDE4 inhibitors, and were subsequently used as APC to stimulate allogeneic T cells. PDE4 inhibitor-matured DC were as effective as vehicle-matured DC in inducing T cell proliferation (Fig. 5). This indicates that DC matured in the presence of PDE4 inhibitors retained their capacity to stimulate T cells.

PDE4 inhibitors modulate the ability of maturing DC to produce inflammatory cytokines, but no longer influence fully mature DC

It has previously been demonstrated that the levels of DC-derived IL-12 are determined during DC maturation and are resistant to further modulation (20,21). Therefore, we studied whether the cytokine-producing capacity of DC matured in the presence of PDE4 inhibitors would be affected. In addition, we questioned whether DC matured in the absence of PDE4 inhibitors would be resistant to subsequent CD40L activation in the presence of PDE4 inhibitors. As depicted in Fig. (6A), DC matured with MF in the presence of PDE4 inhibitors showed a reduced capacity to produce IL-12p70 and TNF-α (40–60% inhibition) upon subsequent stimulation with CD40L-transfected cells in the absence of PDE4 inhibitors. Although both Rolipram and Cilomilast modulate the cytokine-producing capacity of maturing DC, they did not directly affect cytokine production of DC that completed the process of maturation (Fig. 6B). Comparable results were obtained with LPS-matured DC (data not shown).

PDE4 inhibitors present during DC maturation modulate DC-polarizing capacity

As DC matured in the presence of PDE4 inhibitors showed a reduced capacity to produce IL-12p70 upon subsequent CD40L stimulation, we tested whether T_h1/N priming by these DC would be affected. Purified naive CD4+ CD45RA+ T cells were stimulated with SEB presented by different populations of MF-matured DC obtained in the absence (vehicle) or presence of PDE4 inhibitors. Priming of T_h1/N by the PDE4 inhibitor-matured DC modified the T_h1/T_h2 balance and
resulted in a decrease of IFN-γ-producing (T_h1) cells when compared with T cells primed by mature control DC (Fig. 7). Similar reduction of Th1-type producers was observed with DC matured in the presence of LPS and PDE4 inhibitors (data not shown).

**Discussion**

The present study shows predominant cAMP-hydrolyzing activity of the PDE4 family in DC. The PDE4-specific inhibitors Rolipram and Cilomilast suppressed the production of IL-12p70 and TNF-α by immature DC. In contrast, they did not influence the cytokine-producing capacity of fully mature DC. DC exposed to PDE4 inhibitors during maturation showed a reduced ability to produce IL-12p70 and TNF-α, but were not affected in their expression of maturation markers and co-stimulatory molecules nor in their ability to stimulate the proliferation of T_h1 cells. However, priming of naive T_h1 cells by PDE4 inhibitor-exposed DC reduced the development of effector T_h1 cells expressing the T_h1-type cytokine IFN-γ. These data indicate that PDE4 inhibitors exert anti-inflammatory effects on DC which may influence the outcome of the immune response.

We predominantly found enzymatic activities of PDE4 followed by PDE3 and PDE1 in monocytes, immature DC and mature DC, in accordance with the PDE profile previously described for monocytes and other immune cells (3,22–24). The only published study so far that examined PDE activities in human DC as compared to monocytes reported a strong increase in PDE3 activity and a concomitant decrease in PDE4 activity during differentiation of monocytes to DC. However, the inhibitory pattern of the selective PDE inhibitors did not reflect the observed PDE profile, since the PDE4 inhibitor was shown to decrease the TNF-α production by their DC, whereas the PDE3 inhibitor did not have any effect when used alone (22). Although we do not have an explanation for the observed differences in PDE profiles in our study and theirs, we show a similar inhibition of TNF-α production by DC at the same dose of PDE4 inhibitor.

Here, in DC, we demonstrate high expression levels of PDE4A subtype mRNA, lower levels of PDE4B and PDE4D subtype mRNAs, while PDE4C mRNA was lacking. These findings differ from what has been described so far for monocytes and other immune cells that showed predominant...
mRNA expression of the PDE4B subtype followed by A and D (23,25–27). Because PDE4 isoenzyme is widely expressed in many leukocytes and tissues, PDE4-selective inhibitors may produce undesired side effects (3,4). One of the approaches to reduce these side effects is to develop subtype-specific inhibitors. As immature monocyte-derived DC mainly express the PDE4A subtype, it will be interesting to investigate whether different subsets of DC in vivo will show a similar pattern of PDE4 subtype expression.

There have been numerous reports describing the anti-inflammatory properties of PDE4 inhibitors in both in vivo and in vitro settings [reviewed in (3,4,7)]. The inhibition of the cytokines TNF-α and IFN-γ appears to be critical for the anti-inflammatory effect of these potential therapeutic agents. Several studies demonstrated that PDE4 inhibitors suppress TNF-α release by monocytes or macrophages stimulated in vitro (23,28–31). Recently, similar findings were also observed for monocyte-derived DC (22,32) and murine bone marrow-derived DC (33). Our data extend these earlier observations and show that PDE4 inhibitors not only reduce TNF-α production, but also affect the secretion of the Th1-skewing cytokine IL-12 by human DC. In animal models for collagen-induced arthritis (31) and diabetes (34), the PDE4 inhibitor Rolipram inhibited IL-12 production by in vitro stimulated murine macrophages. Furthermore, Rolipram was shown to inhibit superantigen-mediated IL-12 production by human recombinant human PBMC (35). The efficacy of Rolipram to inhibit IL-12 production has been confirmed in vivo in different animal models (34,36). Here we demonstrate that Rolipram and Cimilast exert an inhibitory effect on bioactive IL-12 production by human immature DC. In addition, we show that PDE4 inhibitors affect cytokine production of maturing DC, but not of DC that have completed the process of final maturation. This suggests that PDE4 inhibitors, when present in the lymph nodes, do not have much effect, but strongly influence DC function when present at the site of DC activation.

It has been proposed that the inhibitory effect of Rolipram on murine TNF-α and IL-12 production was partially mediated via up-regulation of IL-10 (33,37,38), but other reports showed that it was independent of IL-10 (36). In our human system, the suppressive effect of PDE4 inhibitors on IL-12 and TNF-α production was probably not mediated by IL-10, as the low levels of IL-10 produced by these DC were not affected. Moreover, a neutralizing anti-IL-10 mAb was unable to reverse the inhibitory effect of Rolipram, Cimilast or db-cAMP on TNF-α production, but also affect the secretion of the Th1-skewing cytokine IL-12 by human DC. In animal models for collagen-induced arthritis (31) and diabetes (34), the PDE4 inhibitor Rolipram inhibited IL-12 production by in vitro stimulated murine macrophages. Furthermore, Rolipram was shown to inhibit superantigen-mediated IL-12 production by human recombinant human PBMC (35). The efficacy of Rolipram to inhibit IL-12 production has been confirmed in vivo in different animal models (34,36). Here we demonstrate that Rolipram and Cimilast exert an inhibitory effect on bioactive IL-12 production by human immature DC. In addition, we show that PDE4 inhibitors affect cytokine production of maturing DC, but not of DC that have completed the process of final maturation. This suggests that PDE4 inhibitors, when present in the lymph nodes, do not have much effect, but strongly influence DC function when present at the site of DC activation.

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Interestingly, low intracellular cAMP levels have been found for patients with multiple sclerosis (41) and rheumatoid arthritis (42). The exact mechanism of cAMP-induced inhibition of IL-12 and TNF-α production is not yet known, but it has been proposed that reduced activation of NF-κB by cAMP-mediated enhancement of the transcription and synthesis of the inhibitor of NF-κB, IkB, is involved (43). Because the human IL-12p40 promoter contains a NF-κB site (44,45) and NF-κB is involved in the positive regulation of TNF-α gene transcription (46) it could be speculated that PDE4 inhibitors down-regulate NF-κB activity in DC. Recently, PDE4 inhibitors were suggested to regulate phosphorylation of cAMP-responsive element (CRE)-binding proteins in mononuclear cells (47). Since the human IL-12p40 promoter contains a CRE-like motif (45) this may be another possible explanation for the cAMP-induced inhibition of IL-12 production.

We show that PDE4 inhibitors decrease inflammatory cytokine production by DC, but we never observed a complete inhibition of TNF-α and IL-12 production. It has been reported previously that PDE3 inhibitors potentiate the inhibitory effects of PDE4 inhibitors, and these two compounds strongly inhibit TNF-α production by DC (22) or T cell proliferation and cytokine production (19) when used in combination. We are thus currently investigating the effects of the combination of PDE3 and PDE4 inhibitors on DC function. Preliminary results indicate that PDE3 inhibitors show no effect on cytokine production on their own, but we observed complete abrogation of CD40L-induced IL-12 and reduced TNF-α production by DC when we simultaneously used PDE4 and PDE3 inhibitors (supplementary data). This suggests that PDE4 and PDE3 are the main PDE enzymes involved in the regulation of DC cytokine production. It is important to note that PDE4 inhibitors had little or no effect on the production of other pro-inflammatory cytokines such as IL-6 and IL-1β by DC (data not shown) and this observation fits with previous reports [reviewed in (3)].

Even though PDE4 inhibitors reduce DC cytokine production, our data show that the presence of PDE4 inhibitors during the maturation of DC did not alter expression of CD83, MHC class II molecule HLA-DR, co-stimulatory molecules or MR. In addition, the capacity to take up antigen by immature DC, either via endocytosis or MR mediated, was not affected by the treatment of immature DC with PDE4 inhibitors (data not shown). However, exposure of DC to PDE4 inhibitors during maturation induced up-regulation of the chemokine receptor CXCR4. Previously, a similar effect on CXCR4 expression was reported for cholera toxin-exposed DC and this effect was partially explained by the ability of cholera toxin to increase intracellular cAMP levels (48). Indeed, we observed that the cAMP-elevating agents db-cAMP and PGE2, similarly to PDE4 inhibitors, induced up-regulation of CXCR4 on DC (C. Moulon, unpublished). This observation will require additional experiments to investigate whether PDE4 inhibitor-treated DC may exhibit a modified response to chemotactic stimuli that are important for their migration towards secondary lymphoid organs (49).

In accordance with the unaffected expression of HLA-DR and co-stimulatory molecules on DC matured in the presence of PDE4 inhibitors, we observed that the capacity to stimulate Tc proliferation by these cells was not influenced. This is in line with a previous report that showed that suppression of T cell proliferation by PDE4 inhibitors was not mediated via pretreatment of APC with PDE4 inhibitors, but rather via their direct effect on T cells (50). Indeed, several other reports describe direct inhibitory effect on T cells by PDE4 inhibitors (19,27). In contrast, others showed reduced ability of PDE4 inhibitor-treated DC to stimulate antigen-specific T cell proliferation (22). However, in that study PDE4 inhibitors were probably acting directly on the T cells as the drug was present during co-culture of DC and T cells.
Histamine, PGE\textsubscript{2}, β\textsubscript{2}-agonists and cholera toxin inhibit IL-12 production by DC and the development of Th\textsubscript{1} cells, by increasing intracellular cAMP (14,20,48,51,52). We now show similar results for PDE4 inhibitors as they inhibited the IL-12-producing capacity of DC and reduced the development of IFN-γ-producing (Th\textsubscript{1}) cells. Since DC, via their production of IL-12, may contribute to the pathology of Th\textsubscript{1}-mediated diseases, it is tempting to speculate that in such situations PDE4 inhibitory effects on IL-12 production by DC may be beneficial. Previously, PDE4 inhibitors have been shown to be effective in animal models for autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (53,54). Recently, Rolipram was shown to reduce the number of TNF-α- and IFN-γ-secreting cells of patients with multiple sclerosis (55). In addition, a non-selective PDE inhibitor was evaluated in patients with multiple sclerosis and was found to inhibit IL-12 production by PBMC; however, more importantly, six out of eight patients reported improved motor skills and less fatigue (56).

In conclusion, we show that PDE4 inhibitors can diminish Th\textsubscript{1} responses in vitro via their reducing effect on IL-12 production by DC. This suggests that in vivo PDE4 inhibitors could amplify their anti-inflammatory effect on T cells by not only acting directly at T cells, but also at the DC level. This may increase our understanding of the therapeutic potential of PDE4 inhibitors for Th\textsubscript{1}-mediated diseases.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>CRE</td>
<td>cAMP-responsive element</td>
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<tr>
<td>db-cAMP</td>
<td>N\textsuperscript{-2’-O-dibutyryl} cyclic AMP</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MF</td>
<td>mannose receptor</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
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<td>PE</td>
<td>phycocythrin</td>
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<tr>
<td>rh</td>
<td>recombinant human</td>
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<tr>
<td>SEB</td>
<td>staphylococcal enterotoxin B</td>
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<tr>
<td>T\textsubscript{h}N</td>
<td>naive Th\textsubscript{1} cell</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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</tbody>
</table>

References

20. Kalinski, P., Schuitemaker, J. H. N., Hilken, C. M. U. and Kapsenberg, M. L. 1998. Prostaglandin E\textsubscript{2} induces the final maturation of IL-12-deficient CD14\textsuperscript{+}CD83\textsuperscript{−} dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. *J. Immunol.* 161:2804.


