Prostaglandin I\(_2\) Analogs Inhibit Proinflammatory Cytokine Production and T Cell Stimulatory Function of Dendritic Cells


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Prostaglandin I$_2$ Analogs Inhibit Proinflammatory Cytokine Production and T Cell Stimulatory Function of Dendritic Cells


Signaling through the PG$_I_2$ receptor (IP) has been shown to inhibit inflammatory responses in mouse models of respiratory syncytial viral infection and OVA-induced allergic responses. However, little is known about the cell types that mediate the anti-inflammatory function of PG$_I_2$. In this study, we determined that PG$_I_2$ analogs modulate dendritic cell (DC) cytokine production, maturation, and function. We report that PG$_I_2$ analogs (iloprost, cicaprost, treprostinil) differentially modulate the response of murine bone marrow-derived DC (BMDC) to LPS in an IP-dependent manner. The PG$_I_2$ analogs decreased BMDC expression of CD86, CD40, and MHC class II molecules by BMDCs and inhibited the ability of BMDCs to stimulate Ag-specific CD4 T cell proliferation and production of IL-5 and IL-13. These findings suggest that PG$_I_2$ signaling through the IP may exert anti-inflammatory effects by acting on DC.

Prostaglandin I$_2$, also known as prostacyclin, is a metabolite of arachidonic acid that has been recently shown to have anti-inflammatory functions (1–3). In a murine respiratory syncytial virus (RSV)$^1$ infection model, we reported that deficiency of the PG$_I_2$ receptor (IP) resulted in increased RSV-induced and inflammation-associated weight loss. In contrast, overexpression of PG$_I_2$ synthase in mouse lung epithelium protected mice from RSV-induced weight loss (1). An immunosuppressive effect of PG$_I_2$ on Th2-mediated inflammation has been also demonstrated with an OVA-induced allergy model (2, 3). IP-deficient mice (IP$^{-/-}$) exhibited increased allergic responses to OVA challenges compared with wild-type mice (IP$^{+/+}$) as indicated by increased leukocyte accumulation and IL-4 and IL-5 production in the airway of the sensitized animals (2, 3). However, the cellular and molecular mechanisms of PG$_I_2$-mediated anti-inflammatory effects remain to be determined.

Dendritic cells (DCs) play important roles in inflammation and in innate and adaptive immune responses to pathogens and nonself substances. They present Ag in the context of MHC molecules to T cells leading to the development of immune responses. In addition, DCs produce a variety of mediators including cytokines and chemokines that may either up- or down-regulate immune and inflammatory responses. Because excessive inflammatory responses may cause tissue damage and be harmful to the host, resolution of inflammation is required for the recovery of normal tissue function and tissue homeostasis. In vivo studies in RSV infection and in OVA-induced allergic responses (1–3) suggest that PG$_I_2$ is one of the mediators that might contribute to the limitation of inflammation. The anti-inflammatory function of PG$_I_2$ may be mediated by direct effects of PG$_I_2$ on DCs that respond to RSV infection and allergens.

PG$_I_2$ exerts its function through the G$_s$ protein-coupled IP and signaling through the IP leads to increased levels of intracellular cAMP (4, 5). Because PG$_I_2$ is very unstable in aqueous solution, PG$_I_2$ analogs, such as iloprost and cicaprost, have been shown to inhibit production of proinflammatory cytokine TNF-$\alpha$ and GM-CSF, while increasing the production of an anti-inflammatory cytokine IL-10 by human peripheral mononuclear cells in vitro (6–9). In another study, the PG$_I_2$ analog treprostinil inhibited production of multiple cytokines including IL-6, TNF-$\alpha$, GM-CSF, and IL-1 by human alveolar macrophages and blocked NF-$\kappa$B nuclear translocation (10). However, the effect of PG$_I_2$ analogs on DCs has not been well-studied. We hypothesized that PG$_I_2$ analogs exert anti-inflammatory activity by suppressing DC activation, maturation, and T cell stimulatory function. To test this hypothesis, we

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$^3$Abbreviations used in this paper: RSV, respiratory syncytial virus; IP, the PG$_I_2$ receptor; DC, dendritic cell; BM, bone marrow; BMDC, BM-derived DC; LTR, long terminal repeat; IBMX, 3-isobutyl-1-methylxanthine; IKK, I$\kappa$B kinase; cIKK2, constitutively active IKK 2; COX, cyclooxygenase; PKA, protein kinase A; CBP, CREB-binding protein.

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examined the in vitro effect of three PGI₂ analogs, iloprost, cicaprost, and treprostinil, on LPS-induced cytokine and chemokine production, maturation, and NF-κB activity of bone marrow (BM)-derived DCs (BMDCs). We used BMDCs of IP⁻/⁻ mice to study the receptor specificity of the effects of PGI₂ analogs. We also examined the effect of PGI₂ analogs on DC ability to stimulate Ag-specific T cell proliferation by using BMDCs and CD4T cells of DO11.10 mice.

Materials and Methods

Mice

IP⁻/⁻ mice were generated by homologous recombination in embryonic stem cells and were backcrossed to the C57BL/6 background for >10 generations (11). Age- and sex-matched C57BL/6 mice (from The Jackson Laboratory) were used as wild-type control mice (IP⁺/⁺). NF-κB reporter transgenic mice, referred to as HLL (HIV-long terminal repeat (LTR)/ luciferase) mice on a C57BL/6/DBA background, were generated as described (12). HLL mice carry the Photinus luciferase gene cDNA driven by the proximal 5' HIV (HIV-1) LTR promoter (12, 13). The proximal HIV-LTR contains two NF-κB motifs and is a well-characterized NF-κB-responsive promoter (14). In primary culture, NF-κB activation is required for transcriptional activity of the proximal HIV-LTR (15, 16). Therefore, luciferase expression in HLL cells is used as a surrogate marker for NF-κB activity. DO11.10 mice that express OVA-specific transgenic TCR specific for OVA peptide 257-264 were obtained from The Jackson Laboratory. In caring for animals, the investigators adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (revised 1996).

Reagents

Cicaprost was a gift from Dr. M. Huebner (Schering, Berlin, Germany). Iloprost was obtained from Cayman Chemicals. Treprostinil was provided by United Therapeutics. Indomethacin, ibuprofen, forskolin, N²-O-dibutyladenosine 3'5'-cyclic monophosphate sodium (db-cAMP), 3-isobutyl-1-methylxanthine (IBMX), and 8-bromoadenosine-3',5'-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS) were purchased from Sigma-Aldrich. Anti-IL-10 mAb and control rat IgG1 were purchased from BD Pharmingen.

Dendritic cells

BMDCs and macrophages were generated using a previously described method with modifications (17). Briefly, the BM in femurs and tibias of IP⁻/⁻, IP⁺/⁺, HLL, and DO11.10 mice was flushed out with RPMI 1640 medium (Mediatech) and a single-cell suspension was prepared by passing the BM solution through a 19-gauge needle five times. After lysis of RBC, the cells were passed through a nylon cell strainer with a mesh size at 70 μm. The cells were then washed with RPMI 1640 medium and resuspended at 5 × 10⁶ cells/ml in complete RPMI 1640 medium containing 5% heat-inactivated FBS (HyClone), 50 μg/ml gentamicin, and 55 μM 2-ML GM-CSF (R&D Systems) was added to the cell solution at 20 ng/ml. The cells were cultured at day 0 in 6-well plates (Corning) and maintained at 37°C in humidified air containing 5% CO₂. On days 3 and 6, half of the culture medium was replaced with fresh complete medium containing 20 ng/ml GM-CSF. At day 8, non- and loosely adherent cells were harvested. Greater than 60% of the harvested cells were CD11c⁺ and >95% CD11b⁺. These cells were either simultaneously activated with LPS (1 μg/ml) and treated with PGI₂ analogs or further purified with anti-murine CD11c magnetic beads (Miltenyi Biotec) according to the manufacturer’s instructions. The purified DCs (purity >94% CD11c⁺ as assessed by flow cytometry) were treated with PGI₂ analogs in the presence of LPS (1 μg/ml). The levels of PGE₂ and stable metabolite of PGI₂, 6-keto-PGF₁α, in the culture supernatant were measured by a modified stable isotope dilution assay that used gas chromatography-negative ion chemical ionization-mass spectrometry as previously described (18).

Cell treatment with PGI₂ analogs and cAMP-elevating agents

BMDCs were treated by adding diluted PGI₂ analogs (cicaprost, iloprost, treprostinil), indomethacin, ibuprofen, and vehicle solutions once directly to the culture medium in the presence of LPS (1 μg/ml). The cells were further cultured for 24 h before the culture supernatant was harvested for cytokine and chemokine measurements and the cells were stained for flow cytometric analyses. Specific concentrations of these agents were used as indicated in Results. BMDCs were also treated with cAMP-elevating agents including db-cAMP (500 μM), forskolin (5 μM), and forskolin plus IBMX (100 μM) for the presence of LPS (1 μg/ml). In some experiments, IL-10 neutralizing Ab (20 μg/ml) or control rat IgG1 (20 μg/ml) were added together with PGI₂ analogs. Because the PGI₂ analogs and cAMP-elevating agents were dissolved in different aqueous solutions or organic solvents, vehicle solutions were diluted in the identical fashion as the respective analogs and agents and used to treat cells with the same protocols as control treatments. The vehicle solutions were: methyl acetate for iloprost, distilled water for cicaprost, db-cAMP, and Rp-8-Br-cAMPS, specifically formulated citrate-buffered solution from United Therapeutics for treprostinil, ethanol for indomethacin and ibuprofen, and DMSO for forskolin and IBMX. For combined treatment with two reagents, cells were pretreated with Rp-8-Br-cAMPS (200 μM) and IBMX (100 μM) for 30 min at 37°C followed by addition of PGI₂ analogs and forskolin (5 μM), respectively.

Cytokine measurement

Multiple cytokines and chemokines (see list in Table I) in the cell culture supernatant were measured by a commercially available LINCOplex Mouse Cytokine/Chemokine kit (LINCO Research) using fluorescently labeled microsphere beads and a Luminex reader. In some experiments, Quantikine and DuoSet ELISA kits (R&D Systems) were used to measure the secretion of cytokines and chemokines (IL-12 p70, TNF-α, GM-CSF, MIP-1α, and MCP-1) according to the manufacturer’s instructions.

CAMP measurement

IP⁻/⁻ and IP⁺/⁺ BMDCs were seeded in 5-ml round-bottom tubes (BD Biosciences) at 1 × 10⁶ cells/tube in 1 ml of complete medium and treated with iloprost, cicaprost, and forskolin in the presence of LPS (1 μg/ml) for 10 min at 37°C. Intracellular cAMP was assayed by using a low pH cAMP assay kit (R&D Systems) according to the manufacturer’s instructions.

Transduction with adenosinergic vectors

A replication-deficient adenosinergic vector that expressed constitutively active Isb kinase 2 (cIKK2) and a control adenosinergic vector that expressed GFP (AdGFP) were constructed as previously described (19). For adenosinergic transduction, purified HLL BMDCs at a concentration of 1 × 10⁶ cells/ml was incubated with cIKK2 or AdGFP (at a multiplicity of infection of 200) at 37°C for 4 h. The cells were washed and cultured in 96-well plates for 12 h before being treated with PGI₂ analogs and cAMP-elevating agents. The efficiency of transduction was indicated by >60% of BMDCs being visibly green under observation through a dissecting microscope 24 h after AdGFP transduction (data not shown).

Luciferase assay

Purified BMDCs of HLL mice were seeded at 1 × 10⁷ cells/ml in complete medium in 96-well plates (200 μl/well) and treated with PGI₂ analogs, cAMP-elevating agents, or vehicle solutions in the presence of LPS (1 μg/ml) for 4 h. HLL BMDCs that had been transduced with either cIKK2 or AdGFP were seeded at 1 × 10⁷ cells/ml for treatments in the absence of LPS for 4 h. Luciferase activity was measured by using the luciferase reporter

### Table I. Cytokine and chemokine production by BMDCs

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Nonstimulated (pg/ml ± SD)</th>
<th>LPS Stimulated (pg/ml ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-1α</td>
<td>285 ± 30</td>
<td>68,494 ± 13,636</td>
</tr>
<tr>
<td>IL-6</td>
<td>37 ± 9</td>
<td>41,849 ± 10,991</td>
</tr>
<tr>
<td>TNF-α</td>
<td>54 ± 1</td>
<td>4,366 ± 1,422</td>
</tr>
<tr>
<td>KC</td>
<td>162 ± 1</td>
<td>4,077 ± 1,431</td>
</tr>
<tr>
<td>RANTES</td>
<td>76 ± 3</td>
<td>1,423 ± 505</td>
</tr>
<tr>
<td>MCP-1</td>
<td>246 ± 15</td>
<td>994 ± 19</td>
</tr>
<tr>
<td>IP-10</td>
<td>377 ± 28</td>
<td>761 ± 137</td>
</tr>
<tr>
<td>G-CSF</td>
<td>13 ± 4</td>
<td>151 ± 29</td>
</tr>
<tr>
<td>IL-1β</td>
<td>11 ± 0</td>
<td>97 ± 11</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>cAMP-elevating agents</td>
<td>0 ± 22</td>
<td>28 ± 11</td>
</tr>
<tr>
<td>IL-10</td>
<td>0</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0</td>
<td>19 ± 0</td>
</tr>
<tr>
<td>IL-9</td>
<td>0</td>
<td>17 ± 0</td>
</tr>
</tbody>
</table>

* Purified CD11c⁺ BMDCs were treated without or with LPS (1 μg/ml) for 24 h.
The levels of cytokines and chemokines in the culture supernatant were measured by a Luminex assay system with a LINCOplex kit.
Flow cytometric analysis

IP-1 and IP-5 BMDCs were treated with iloprost and vehicle solutions for 24 h. Non- and loosely adherent cells were harvested for staining with fluorochrome-labeled Abs against CD11b, CD11c, PDGFRa, CD40, and MHC class II (I-A<sup>B</sup>; BD Pharmingen) and analyzed using a LSR II flow cytometer (BD Biosciences). A total of 10,000 live cell events as gated on forward and side scatter characteristics was acquired. CD86, CD40, and MHC class II expression were analyzed on a live DC population gated on CD11b and CD11c double-positive cells.

Stimulation of CD4<sup>+</sup> T cells by DCs

CD4<sup>+</sup> T cells were purified from the spleens of DO1.10 mice with antimurine CD4 magnetic beads (Miltenyi Biotec) according to the manufacturer’s instructions. The cells were labeled with 5 μM CFSE (Molecular Probes) in PBS for 10 min at room temperature. The reaction was quenched with PBS containing 25% serum. The cells were washed twice with complete medium before being cocultured with DCs.

BMDCs of DO1.10 mice were treated with iloprost (400 nM), cicaprost (400 nM), or vehicle solutions in the presence of LPS (1 μg/ml) and 100 μg/ml OVA (Sigma-Aldrich) for 24 h. The DCs were washed with medium three times and specific numbers of DCs (5,000 and 20,000 cells) in each PGI<sub>2</sub> analog- or vehicle-treated group were used for coculture with CFSE-labeled CD4<sup>+</sup> T cells (2 × 10<sup>4</sup> cells) for 4 days. The cells were analyzed for CFSE fluorescence intensity by flow cytometry. A reduction of CFSE intensity by half is an indication of a cell division. The cells were also subjected to viable cell counting using a Guava PCA 96 system and Guava ViaCount Flex Reagent (Guava Technologies). The culture supernatant was used for IL-5 and IL-13 measurement.

Statistics

The p values were calculated by using the unpaired Student t test. Values of p < 0.05 were considered significant.

Results

PGI<sub>2</sub> analogs decrease the production of multiple proinflammatory cytokines and chemokines while increasing the production of IL-10 by BMDCs

To test the hypothesis that PGI<sub>2</sub> analogs down-regulate proinflammatory cytokine production by inhibiting activation of DCs, we differentiated BM precursor cells of C57BL/6J IP<sup>-/-</sup> mice to BMDCs and macrophages in GM-CSF for 8 days, then activated the mixed cell population with LPS and treated them with exogenous PGI<sub>2</sub> analogs including iloprost, cicaprost, and treprostinil for 24 h. The cell culture supernatant was assayed for cytokine production. To examine the effect of endogenous PGs on the function of BMDCs, we treated the cells with a nonselective cyclooxygenase (COX) inhibitor, indomethacin, to inhibit endogenous prostaglandin production. The cells differentiated in GM-CSF for 8 days expressed the IP as analyzed by RT-PCR (data not shown, primer sequences were: forward, 5′-CCGCGC AACAG AGACCG CCACC AT-3′; reverse, 5′-CGGGC ACACAG GCAAC ACAAC CA-3′). Treatment of these cells with the three PGI<sub>2</sub> analogs significantly inhibited the production of LPS-induced proinflammatory cytokine IL-12 p70 in a dose-dependent pattern (Fig. 1). In contrast, the three analogs dose-dependently increased the production of anti-inflammatory IL-10 (Fig. 1). In the culture supernatant of the cells stimulated with LPS, PGE<sub>2</sub> was measured to be <1 nM and the stable metabolite of PGI<sub>2</sub>, 6-keto-PGF<sub>1</sub>α, was below the limit of detection. Inhibition of endogenous PGs with indomethacin at 40 and 400 nM did not change the production of these cytokines (Fig. 1, only showing the data for 400 nM indomethacin). The lack of effect by the COX inhibitor indomethacin on BMDC cytokine production was confirmed by the use of an alternative COX inhibitor, ibuprofen, at the same doses (data not shown). These data indicate that the endogenously produced PGs did not have a measurable overall impact on the cytokine production.

To examine the specific effect of PGI<sub>2</sub> analogs on DCs, we purified DCs (CD11C<sup>+</sup>) cells from the BM-derived cell population. LPS stimulation of the purified BMDCs induced the production of multiple cytokines and chemokines (Table I). The levels of IL-4, IL-5, IL-7, IL-13, IL-15, IL-17, and IFN-γ were under the limit of detection. PGI<sub>2</sub> analogs, iloprost, cicaprost, and treprostinil, significantly decreased LPS-induced production of IL-12, TNF-α, IL-1α, IL-6, MIP-1α, and MCP-1, but increased the production of IL-10 (Fig. 2A). Because IL-10 has been shown to be an inhibitor of proinflammatory cytokine expression and exogenously added IL-10-suppressed IL-12 and TNF-α production by BMDCs (20–23), the inhibitory effect of PGI<sub>2</sub> analogs on the proinflammatory cytokine and chemokine production might have been mediated by the concurrent elevation of IL-10 expression. To examine this possibility, an IL-10-specific neutralizing Ab was added to the culture during the period of treatment with PGI<sub>2</sub> analogs. Neutralization of IL-10 did not abrogate the inhibitory effect of PGI<sub>2</sub> analogs (Fig. 2B), although addition of anti-IL-10 to cicaprost-treated BMDC culture resulted in an undetectable level of IL-10 in the culture supernatant (Fig. 2B). These data indicate that the PGI<sub>2</sub> analog-mediated inhibition is not a result of concurrent stimulation of IL-10 expression.

IP-dependent inhibitory effect of PGI<sub>2</sub> analogs on DC cytokine and chemokine production

We next investigated the role of IP signaling in PGI<sub>2</sub>-induced changes of cytokine and chemokine production by using IP<sup>-/-</sup> and IP<sup>+/+</sup> DCs. As demonstrated in Fig. 3, treatment of purified IP<sup>-/-</sup> BMDCs with the PGI<sub>2</sub> analogs iloprost (Fig. 3A) and cicaprost (Fig. 3B) dose-dependently inhibited LPS-induced production of IL-12, TNF-α, IL-1α, IL-6, MIP-1α, and MCP-1; the PGI<sub>2</sub> analogs up-regulated the production of IL-10 as compared with the appropriate vehicles. In contrast, treatment of IP<sup>+/+</sup> DCs with iloprost and cicaprost at the same concentrations did not significantly affect...
FIGURE 2. The effect of PGI₂ analogs and neutralizing anti-IL-10 on the cytokine and chemokine production by DCs. A. Purified BMDCs were treated with indomethacin (40 nM) or PGI₂ analogs including iloprost (40 nM), cicaprost (10 nM), and treprostinil (40 nM) in the presence of LPS for 24 h. B. BMDCs were treated with LPS and cicaprost (40 nM) in the presence of neutralizing anti-IL-10 (20 μg/ml) or rat IgG1 control for 24 h. The levels of multiple cytokines and chemokines in the culture supernatants were measured by a Luminex assay system with a LINCoplex kit. Representative data (mean ± SD) of (A) four or (B) three experiments. *, p < 0.05 compared with vehicle-treated cells.

FIGURE 3. The IP-dependent modulatory effect of PGI₂ analogs on the cytokine and chemokine production. IP<sup>−/−</sup> and IP<sup>+/−</sup> BMDCs were treated with (A) iloprost (0.4, 40 nM), and (B) cicaprost (0.1, 10 nM) in the presence of LPS for 24 h. The levels of multiple cytokines and chemokines were measured by a Luminex assay system with a LINCoplex kit. Representative data (mean ± SD) of three experiments. *, p < 0.05 compared with vehicle-treated cells.

LPS-induced production of any of these cytokines and chemokines compared with vehicle treatments, indicating an essential role of IP-dependent signaling in PGI₂ analog-mediated modulation of the cytokine and chemokine production.

**PGI₂ analogs increase the levels of cAMP and decrease NF-κB activity in BMDCs**

Stimulation of the IP leads to increased intracellular cAMP levels (24, 25). To examine the effect of IP signaling on cAMP production in DCs, purified BMDCs were treated with cicaprost and iloprost in the presence of LPS for 10 min and analyzed for intracellular cAMP production. As shown in Fig. 4A, iloprost and cicaprost significantly elevated intracellular cAMP levels up to 10- and 6-fold, respectively, in IP<sup>−/−</sup> BMDCs, but not in IP<sup>+/−</sup> BMDCs. These data suggest that the modulatory effect of PGI₂ analogs on cAMP production was dependent on IP signaling. Forskolin induced similar levels of cAMP in both IP<sup>−/−</sup> and IP<sup>+/−</sup> BMDCs, indicating that IP<sup>−/−</sup> BMDCs were able to generate a cAMP response to non-IP-dependent stimulation.

To further investigate whether the effect of PGI₂ analogs on DC cytokine and chemokine production was mediated by cAMP signaling, we used Rp-8-Br-cAMPS to specifically inhibit a cAMP-dependent protein kinase, protein kinase A (PKA). PKA is an important mediator in the cAMP-signaling pathway and has been implicated in a wide range of cellular processes. We incubated IP<sup>−/−</sup> BMDCs in the presence or absence of Rp-8-Br-cAMPS for 30 min before the treatment with iloprost or cicaprost. As shown in Fig. 4B, preincubation of the DCs with the PKA inhibitor resulted in 2- and 3-fold increases in TNF-α production, and 4- and 6-fold increases in MIP-1α production by the cells treated with cicaprost and iloprost, respectively. This suggests the PKA inhibitor significantly attenuated the effect of iloprost and cicaprost on the cytokine production. However, under the PKA-inhibited condition, the PGI₂ analog-treated cells still produced significantly less TNF-α and MIP-1α compared with appropriate vehicle-treated cells. Cells treated with iloprost in the presence of Rp-8-Br-cAMPS produced 3.7-fold less TNF-α than vehicle-treated cells (1.1 ± 0.175 ng/ml vs 4.1 ± 0.38 ng/ml, p < 0.05) and 7-fold less MIP-1α (9.4 ± 2.0 ng/ml vs 66.4 ± 6.0 ng/ml, p < 0.05). Cells treated with cicaprost in the presence of Rp-8-Br-cAMPS produced 6.3-fold less TNF-α than vehicle-treated cells (0.74 ± 0.065 ng/ml vs 4.7 ± 0.175 ng/ml, p < 0.05) and 11.7-fold less MIP-1α (6.3 ± 1.1 ng/ml vs 73.6 ± 2.0 ng/ml, p < 0.05). These data indicate that the PKA inhibition did not fully abrogate the effect of the PGI₂ analogs and suggest that the effect of iloprost and cicaprost were partially dependent on PKA-mediated signaling. Alternatively, because Rp-8-Br-cAMPS discriminates between the two isozymes of PKA and prefers type I PKA (26), this PKA inhibitor may not have sufficient potency to
inhibit type II PKA which may be functional in the cAMP-signaling pathway.

It has been shown that NF-κB plays a critical role in DC activation and expression of proinflammatory cytokines and chemokines (27). To test the hypothesis that the anti-inflammatory effect of PGI2 was associated with inhibition of NF-κB activation, we examined the effect of PGI2 analogs on NF-κB transcriptional activity in BMDCs that were derived from NF-κB reporter HLL mice. In cells from these mice, luciferase expression can be assayed as a measure of NF-κB activity (12, 13).

Treatment of purified HLL BMDCs with PGI2 analogs in the presence of LPS significantly decreased LPS-induced luciferase activity (Fig. 4C), suggesting an inhibitory effect of the analogs on NF-κB activity. Treatment of the DCs with indomethacin did not change NF-κB activity. Treatment of cells with a cAMP analog, db-cAMP, and cAMP-elevating agents, IBMX plus forskolin, also resulted in similar reduction of luciferase expression (Fig. 4C), suggesting that up-regulation of intracellular cAMP inhibits NF-κB activity. Cicaprost, db-cAMP, and IBMX plus forskolin also inhibited cIKK2-induced luciferase activity.

FIGURE 5. PGI2 analogs inhibit CD86, CD40, and MHC class II expression on BMDCs. The mixed population of BMDCs and macrophages of IP-/- mice were treated with iloprost or cicaprost at 4, 40, and 400 nM for 24 h. The cells were stained with fluorochrome-labeled Abs specific for CD11b, CD11c, CD86, CD40, and MHC class II and analyzed by flow cytometry. A. The data shown were gated for live, CD11b<sup>+</sup>, and CD11c<sup>+</sup> cells. B. The number of CD86<sup>high</sup>, CD40<sup>high</sup>, or MHC class II<sup>high</sup> cells (M1 gate in A) were presented as percentages of CD11b<sup>+</sup>CD11c<sup>+</sup> double-positive cells (mean ± SD). Representative data of three experiments. *, p < 0.05 compared with vehicle-treated cells.

FIGURE 4. PGI2 analogs increase intracellular cAMP production and suppress NF-κB activity. A. IP<sup>+/+</sup> and IP<sup>−/−</sup> BMDCs were treated with iloprost or cicaprost at 4, 40, 400 nM, with vehicle solutions, or with forskolin at 5 μM in the presence of LPS for 10 min. Cell lysate was used for cAMP assays.

B. IP<sup>−/−</sup> BMDCs were preincubated with Rp-8-Br-cAMPS (200 μM) or mock solution for 30 min followed by treatment with iloprost or cicaprost at 4 nM for 24 h. Supernatant was used for cytokine and chemokine measurements by a Luminex system. C. BMDCs of HLL mice (NF-κB reporter mice) were treated with indomethacin, iloprost, cicaprost, or treprostinil (Trep) at 40 nM in the presence of LPS for 6 h. Alternatively, HLL BMDCs were transduced with adenoviral vector expressing cIKK2, and then treated with cicaprost for 6 h. Cell lysate was used for luciferase assays. D. HLL BMDCs were pretreated with Rp-8-Br-cAMPS at 200 μM for 30 min followed by treatment with cicaprost and iloprost in the presence of LPS for 24 h. Cell lysate was used for luciferase assays. Representative data (mean ± SD) of three experiments. *, p < 0.05 compared with vehicle- (A and C) or mock-treated cells (B and D).
by flow cytometry after treatment of IP
we measured cell surface expression of CD86, CD40, and MHC
increased luciferase activity by 25–42% (Fig. 4
BMDCs with the PKA inhibitor, Rp-8-Br-cAMPS, significantly
activate naive CD4 T cells. DO11.10 BMDCs were
tivated with iloprost or cicaprost at 400 nM for 24 h
PGI2 analogs inhibit the ability of BMDCs to stimulate
Ag-specific T cell proliferation
Based on the inhibition of BMDC activation and maturation by
PGI2 analogs, we hypothesized that BMDCs treated with iloprost and cicaprost would have decreased ability to activate naive CD4 T cells in an Ag-specific fashion. To test this hypothesis, we treated DO11.10 BMDCs with iloprost (400 nM), cicaprost (400 nM), or vehicle solutions in the presence of LPS (1 µg/ml) and OVA (100 µg/ml) for 24 h. The cells were washed three times and cocultured with freshly purified and CFSE-labeled DO11.10 CD4 T cells for 4 days. We found that treatment of BMDCs with OVA was necessary to stimulate DO11.10 CD4 T cell proliferation and the production of IL-5 and IL-13, indicating that the T cell response was OVA specific. Treatment of the BMDCs with iloprost or cicaprost in the presence of OVA and LPS markedly decreased their ability to stimulate DO11.10 CD4 T cell proliferation as indicated by a significant decrease in the number of divided CFSE-labeled T cells (Fig. 6A) and significantly reduced numbers of total T cells in the culture (Fig. 6B), compared with vehicle-treated DCs. In addition, treatment of the BMDCs with iloprost and cicaprost led to decreased IL-5 and IL-13 production by the CD4 T cells (Fig. 6C). Therefore, iloprost and cicaprost not only inhibited the expression of CD86, CD40, and MHC class II, but also suppressed the ability of BMDCs to stimulate T cells in an Ag-specific manner.

Discussion
DCs are the most potent APCs that bridge innate and adaptive immunity (28). Activation and maturation of DCs, in the setting of inflammation, has a profound impact on host defense against pathogens and environmental exposures. In this study, we demonstrated that PGI2 analogs signal through IP to inhibit the production of multiple proinflammatory cytokines and chemokines, while increasing the production of the anti-inflammatory cytokine IL-10 by BMDCs. The suppressive effect of PGI2 analogs on BMDCs was associated with IP-dependent elevation of intracellular cAMP and a decrease of NF-κB activity. Furthermore, these analogs also suppressed DC maturation and function by decreasing CD86, CD40, and MHC class II expression and by inhibiting DC-induced T cell proliferation and cytokine production. These findings suggest that the previously described anti-inflammatory activity of PGI2 and IP signaling (1–3) may involve inhibition of DC activation, maturation, and function.

Treatment of BMDCs with PGI2 analogs resulted in differential effects on cytokine and chemokine expression. All three PGI2 analogs, iloprost, cicaprost, and treprostinil, dose-dependently inhibited the production of IL-12, TNF-α, IL-1, IL-6, MIP-1α, and MCP-1, but increased IL-10 production by the BMDCs, consistent with the finding of Jozefowski et al. (29) that iloprost decreased IL-12, TNF-α, and IL-6 production by BM-derived cells. The cytokines and chemokines that were suppressed by the PGI2 analogs inhibit the ability of BMDCs to stimulate Ag-specific T cell proliferation.
analogs have a variety of functions in inflammatory and immune responses. They may induce fever and acute phase response proteins (IL-1α, IL-6, and TNF-α) (30–32), drive Th1 cell differentiation and cell-mediated immune response (IL-12) (33), and recruit macrophages and monocytes to inflamed areas (MIP-1α and MCP-1) (34). In contrast, IL-10 plays a major role in suppressing inflammatory responses by inhibiting the expression of multiple proinflammatory cytokines (35) and by limiting Th1 immune responses (36). Therefore, PGI₂ analogs inhibited the production of proinflammatory cytokines and chemokines while increasing IL-10 production, suggesting an overall anti-inflammatory effect on BMDCs.

What are the molecular mechanisms of PGI₂ analog-mediated modulation of the production of inflammatory mediators by DCs? Activation of the IP has been shown to elevate intracellular cAMP, an important second messenger, in smooth muscle cells (24) and macrophages (25). Increased cAMP leads to activation of PKA and phosphorylation of the cAMP response element binding protein (CREB), which binds to the CREB element in the target genes for transcription activation (37). It has been shown that induction of cAMP has been associated with the modulatory effects of cAMP-elevating agents including db-cAMP, forskolin, and the phosphodiesterase inhibitor IBMX. These reagents inhibited IL-12, TNF-α, and MIP-1α, while increasing IL-10 production by BMDCs (22, 38, 39). A similar effect of cAMP-elevating agents has been reported on BM-derived macrophages (40). We found that iloprost and cicaprost IP-dependently increased cAMP production in BMDCs in a dose-dependent pattern. The elevated cAMP was associated with the IP-dependent inhibition of the proinflammatory cytokine and chemokine production, and CD86, CD40 and MHC class II expression. The effect on cytokine production was partially attenuated by Rp-8-Br-cAMPS, an inhibitor of PKA in the cAMP-signaling pathway. These data suggest an involvement of the cAMP-signaling pathway in the PGI₂ analog-mediated effect on DCs. The IP-dependent effect of PGI₂ analogs on the production of cAMP and secretion of cytokines and chemokines suggests an important role of IP signaling in the anti-inflammatory function of PGI₂.

Another important finding of this study was that PGI₂ analogs suppressed NF-κB activity in BMDCs. NF-κB is a critical transcription factor in the molecular regulation of inflammatory cytokine and chemokine expression (27). Known NF-κB-dependent genes include IL-12, TNF-α, IL-6, MCP-1, KC, and IL-1β (12, 27, 41, 42). Elevation of intracellular cAMP by cAMP-elevating agents decreased LPS-induced NF-κB function as shown in this study and in several other studies (37, 43–45) and this effect was partially attenuated by Rp-8-Br-cAMPS, suggesting that PGI₂ analogs may suppress NF-κB function by activating the cAMP-signaling pathway. Although the mechanism of NF-κB inhibition by cAMP remains to be investigated, it is possible that elevated levels of cAMP result in more activated CREB. Because both transcription factors, CREB and NF-κB, can use CREB-binding protein (CBP) as coactivator (46), more activated CREB may compete with NF-κB for a limited amount of CBP, leading to decreased NF-κB activity. This possibility is strongly supported by a recent study showing that activation of CREB resulted in up-regulation of CREB-dependent IL-10 production and suppression of NF-κB-dependent expression of inflammatory cytokines (IL-12, TNF-α, IL-6) by human monocytes (47). PGI₂ analogs were capable of inhibiting NF-κB activity in cells expressing the constitutively active form of IKK2, suggesting that a process downstream of IKK2 activity in the NF-κB signaling pathway, possibly involving CBP, was affected. Other possible mechanisms for PGI₂ analog/cAMP-mediated NF-κB inhibition include blocking nuclear translocation of NF-κB (10) and decreasing degradation or up-regulation of IkB (48). The action of PGI₂ analogs makes them effective inhibitors of proinflammatory cytokines and chemokines and our data indicate that this inhibitory effect of PGI₂ analogs is associated with the decrease of NF-κB activity.

PGE₂ is another PG that has anti-inflammatory properties (22, 38, 49). Similar to the effect of PGI₂ analogs, PGE₂ has been shown to inhibit IL-12, TNF-α, and IL-6 production by BMDCs, suppress MHC class II expression, and enhance IL-10 production (22, 29, 38, 39). Studies showed that DCs may be activated to produce high levels of proinflammatory cytokines and chemokines and remain at sites of inflammation or develop into CCR7-expressing cells that allow them to migrate to the draining lymph nodes and induce Ag-specific immune responses (50, 51). These migratory DCs produce low levels of inflammatory cytokines (50). PGE₂ promotes the generation of the migratory DCs, inhibits proinflammatory cytokine production (50), and up-regulates CCR7 expression (52, 53). For generation of migrating DCs, PGE₂ is required at early time points of DC maturation (53). The stimulatory effect of PGE₂ on DC migration is dependent on the signaling through EP2 and/or the EP4 receptor (53, 54). The effect of PGE₂ is believed to be mediated by cAMP pathway because activation of EP2 and EP4 receptor signaling results in elevated levels of intracellular cAMP (55, 56). Although it remains to be determined whether PGI₂ analogs also affect the commitment of DCs to migratory DCs, the similar anti-inflammatory function of PGI₂ and PGE₂ suggest that these PGs produced in inflammatory sites may act together to modulate the inflammatory and immune processes through the cAMP-signaling pathway.

Our in vitro data indicate that endogenous PGs seem not to contribute to the modulation of cytokine and chemokine production by DCs in an autocrine manner. Our result is consistent with the finding of Jozefowski et al. (29). DCs may be affected in vivo by PGs produced by neighboring cells at the sites of inflammation, leading to decreased DC activation, maturation, and function. In the model of OVA-induced allergic inflammation in the lung, multiple PGs including PGI₂, PGE₂, PGF₂α, and PGE₃ were detected in the bronchoalveolar lavage fluid (57–59). The source of PGI₂ may be endothelial cells that constitutively express PGI₂ synthase (60). Macrophages in the lung may be another source of PGI₂ (61). Because of the instability of PGI₂, the physiologic concentration of PGI₂ in the site of inflammation is difficult to determine. However, considering that iloprost exhibited an equilibrium dissociation constant for the IP with a Kᵣ value of 9.8 nM (62), and PGI₂ stimulated adenylyl cyclase activity with an EC₅₀ (the concentration that leads to 50% maximal response) at 6.6 nM in NC8-20 cells (63), it may be reasonable to estimate physiologically functional levels of PGI₂ in a range of 10–100 nM. Therefore, our in vitro data showing the effect of iloprost and cicaprost at a range of 4–400 nM on cytokine expression, cAMP production, and T cell stimulatory function of DCs appear to have a physiological relevance.

In summary, we have demonstrated that PGI₂ analogs suppress the production of multiple proinflammatory cytokines and chemokines, and increase the anti-inflammatory cytokine IL-10 production by BMDCs. The analogs also inhibit DC maturation and their function to activate naive CD4 T cells in an Ag-specific manner. The modulatory effects were associated with elevated intracellular cAMP production and decreased NF-κB activity, suggesting an involvement of cAMP and NF-κB-signaling pathways in mediating the PGI₂ analog-induced effects. The IP-dependent modulatory effect of PGI₂ analogs on DC activation and maturation suggest that PGI₂ may exert its anti-inflammatory function by acting on DCs via an IP-mediated signaling pathway.
Disclosures

The authors have no financial conflict of interest.

References


15. Cytokine 11: 127–133.


