Effect of Prostaglandin I\(_2\) Analogs on Cytokine Expression in Human Myeloid Dendritic Cells via Epigenetic Regulation

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Abstract

Prostaglandin I\(_2\) (PG\(_I_2\)) analog is regarded as a potential candidate for treating asthma. Human myeloid dendritic cells (mDCs) play a critical role in the pathogenesis of asthma. However, the effects of PGI\(_2\) analog on human mDCs are unknown. In the present study, circulating mDCs were isolated from six healthy subjects. The effects of PGI\(_2\) analogs iloprost and treprostinil on cytokine production, maturation and T-cell stimulatory function of human mDCs were investigated. Tumor necrosis factor (TNF-\(\alpha\)) and interleukin (IL)-10 were measured by enzyme-linked immunosorbent assay. The expression of costimulatory molecules was investigated by flow cytometry. T-cell stimulatory function was investigated by measuring interferon (IFN)-\(\gamma\), IL-13 and IL-10 production by T cells cocultured with iloprost-treated mDCs. Intracellular signaling was investigated by Western blot and chromatin immunoprecipitation. We found that iloprost and treprostinil induced IL-10, but suppressed TNF-\(\alpha\) production in polynosinic-polycytidylic acid (poly I:C)-stimulated mDCs. This effect was reversed by the l-prostanoid (IP), E-prostanoid (EP) receptor antagonists or intracellular free calcium (Ca\(^{2+}\)) chelator. Forskolin, an adenyl cyclase activator, conferred a similar effect. Iloprost and treprostinil increased intracellular cyclic monophosphate (cAMP) levels, and iloprost also increased intracellular Ca\(^{2+}\). Iloprost suppressed poly I:C-induced mitogen-activated protein kinase (MAPK) phospho-p38 and phospho–activating transcription factor (ATF)2 expression. Iloprost downregulated poly I:C-induced histone H3K4 trimethylation in the TNFA gene promoter region via suppressing translocation of histone 3 lysine 4 (H3K4)-specific methyltransferases MLL (mixed lineage leukemia) and WDR5 (WD repeat domain 5). Iloprost-treated mDCs inhibited IL-13, IFN-\(\gamma\), IL-10 production and in vivo study, circulating mDCs were isolated from six healthy subjects. The effects of PGI\(_2\) analogs iloprost and treprostinil on cytokine expression through the IP/EP2/EP4 receptors–cAMP and EP1 receptor–Ca\(^{2+}\) pathway. Iloprost suppressed TNF-\(\alpha\) expression via the MAPK-p38-ATF2 pathway and epigenetic regulation by downregulation of histone H3K4 trimethylation.

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INTRODUCTION

Asthma is a chronic airway inflammatory disorder with accumulation of inflammatory cells including eosinophils, lymphocytes, neutrophils and mast cells. The disease process is regulated by the cytokines and chemokines and also by the interactions between the antigen-presenting cells and T cells (1). Tumor necrosis factor (TNF)-\(\alpha\), a pleiotropic proinflammatory cytokine, is increased in TNF-\(\alpha\) mRNA and protein levels in the airways of asthmatic patients (2). Emerging evidence suggests the central role of TNF-\(\alpha\) in asthma for its properties of developing mast cell–mediated airway hyperresponsiveness, activating eosinophil proliferation and regulating chemokine production in monocytes (3). Recent studies suggest the particular role...
of TNF-α in severe refractory asthma according to its properties of neutrophil recruitment, induction of resistance to steroid and involvement of airway remodeling (4). Interleukin (IL)-10 is a broad antiinflammatory cytokine functioning as a feedback regulation of T helper (Th) 1 and Th2 responses (5). IL-10 inhibits survival and cytokine production of inflammatory cells and can limit allergic airway inflammation and hyperreactivity (6). IL-10-deficient mice express highly elevated levels of Th2 cytokine after allergen challenging and exhibit exaggerated airway inflammation (7). In contrast to TNF-α, the level of IL-10 in the lungs of asthmatic patients is significantly decreased (8).

Dendritic cells (DCs) are professional antigen-presenting cells and are highly heterogeneous in terms of origin, morphology, phenotype and function. DCs play a major role in initiation and regulation of adaptive immune responses to the stimulation of antigens and allergens (9). In a murine asthma model, myeloid dendritic cells (mDCs) accumulate in the allergen-challenged airways during the acute phase, and the depletion of mDCs attenuates the airway inflammation and hyperresponsiveness (10). In human asthma, mDCs accumulate in bronchoalveolar lavage fluid after an allergen challenge (11), and the influx of mDCs into the airways can be augmented by endotoxins (12). The induction and maintenance of inflammatory responses to allergens in persistent airway disease needs the involvement of mDCs (13). These data suggested the critical role of mDCs in allergic airway inflammation.

Prostaglandins are generally regarded as proinflammatory molecules. However, prostaglandin I2 (PGI2) was recently shown to exhibit some antiinflammatory properties. Iloprost, a stable PGI2 analog, is a potent vasoconstrictor by iloprost suppresses the cardinal features of asthma via inhibition of lung DC maturation and migration to regional lymph nodes (15). Our previous work demonstrated that iloprost can modulate cytokine expression via the IP receptor in human plasmacytoid DCs (16). However, the effects of PGI2 analogs on human mDCs are still not elucidated.

Epigenetic regulation, including acetylation of core histones by histone acetyltransferase or histone deacetylase, has been shown to be involved in inflammatory expression in monocytes and macrophages (17). In asthmatic patients, the histone acetyltransferase activity is markedly increased, whereas the histone deacetylase activity is reduced, resulting in the overexpression of inflammatory genes (18,19). Recently, we showed that epigenetic regulation is an important mechanism by which iloprost modulates asthma-related chemokines expression in monocytes (20). In the present study, we examined the in vitro effect of two commonly used PGI2 analogs, iloprost and treprostinil, on the expression of cytokines by mDCs and also investigate the intracellular mechanism including epigenetic regulation. The effects of PGI2 analogs on the expression of costimulatory molecules and the T-cell stimulatory functions of mDCs were also studied.

MATERIALS AND METHODS

Isolation and Culture of mDCs

The study protocol was approved by the Institution Review Board of Kaohsiung Medical University Hospital. Peripheral blood samples (250 mL) were obtained from healthy and nonsmoking individuals who had no history of allergic or systemic disease (n = 6) after gaining informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll-Histopaque (Pharmacia Biotech, Uppsala, Sweden) and then separated into a low-density fraction enriched in DCs by centrifuging for 30 min at 300g. Blood mDCs were magnetically sorted from PBMCs using blood DC antigen (BDCA-1) cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s instructions. Isolated mDCs were >90% in purity. Purified mDCs were cultured in 24-well round-bottom plates (105/well) in 500 μL RPMI 1640 buffered with NaHCO3, containing 10% heat-inactivated endotoxin-tested fetal calf serum, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. Isolated mDCs were treated with varying doses of iloprost (10–8 to 10–7 mol/L) or treprostinil (10–9 to 10–7 mol/L) or vehicle solution for 24 or 48 h. In some cases, mDCs were pretreated with varying doses of iloprost (10–8 to 10–7 mol/L) or treprostinil (10–9 to 10–6 mol/L) for 2 h and were stimulated with polyinosinic-polycytidylacid (poly I/C; 10 μg/mL) for 6, 24 or 48 h without the washout of the PGI2 analogs. Supernatants were collected for IL-10 and TNF-α measurement. For the experiment of DC/T cell coculture, mDCs were treated with iloprost (10–9 to 10–7 mol/L) for 48 h and were washed with phosphate-buffered saline (PBS) for three times before being cocultured with T cells.

To examine the involvement of the IP receptor, E-prostanoid (EP) receptor and peroxisome proliferator–activated receptors (PPARs) in the effects of PGI2 analogs, mDCs were pretreated with the IP receptor antagonist (CAY 10449), EP1 receptor antagonist (GW6741), EP3 receptor antagonist (AH6809), EP4 receptor antagonist (GW627368X), PPAR-α antagonist (GW6741) or PPAR-γ antagonist (GW 9662) at the concentration of 10–5 to 10–6 mol/L either alone or 1 h before the treatment of the cells with iloprost or treprostinil and then were treated with or without poly I/C 2 h after iloprost or treprostinil treatment. All IP receptor, EP receptor and PPAR antagonists were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). To examine the involvement of intracellular calcium (Ca2+) in the effects of iloprost, mDCs were pretreated with the intracellular free calcium chelator BAPTA-AM (Sigma-Aldrich, St. Louis, MO, USA) at the concentration of 10–5 to 10–6 mol/L.
either alone or 15 min before iloprost treatment and were then treated with or without poly I:C 2 h after iloprost treatment. In some cases, mDCs were treated with forskolin, an adenyl cyclase activator, for 24 h or pretreated with forskolin for 2 h and then stimulated with poly I:C for 24 h. To investigate the cell signaling, the cells were pretreated with mitogen-activated protein kinase (MAPK)-p38 inhibitor (SB203580), MAPK-JNK (Jun NH2-terminal kinase) inhibitor (SP600125) or MAPK-ERK (extracellular signal–related kinase) inhibitor (PD98059) for 1 h and were stimulated with poly I:C for 24 h. The concentration used in experiments is according to the half maximal inhibitory concentration (IC50) of each MAPK inhibitor and previous studies (21,22). All MAPK inhibitors were purchased from Cayman Chemical Company. Supernatants were collected for IL-10- and TNF-α measurement.

Intracellular Ca2+ Measurements

Intracellular Ca2+ levels were measured using Fluo-3-acetoxymethylester (Fluo-3-AM) as our previous work (23). Briefly, human mDCs were washed with Ca2+-free PBS and then incubated with Fluo-3-AM (5 μmol/L) for 30 min. After being washed with Ca2+-free PBS, mDCs were treated with iloprost (10−7 mol/L) for 2 h and washed and resuspended in calcium-free PBS. The fluorescence intensities of Fluo-3-AM, which reflect the levels of intracellular Ca2+ levels, were measured using flow cytometry.

cAMP Assay

A commercial 3′,5′-cyclic monophosphate (cAMP) enzyme immunoassay kit (Sigma-Aldrich) was used for intracellular cAMP measurement. Human mDCs (1 × 106) were incubated with or without iloprost or treprostinil (10−4 to 10−7 mol/L) for 2 h or pretreated with or without the MAPK-p38 inhibitor SB203580 (5 μmol/L) for 1 h and were stimulated with 10 μg/mL poly I:C for 1 h. The cells were washed with iced PBS once and then resuspended. The cells were lysed in 10 mmol/L HEPES, pH 7.9, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 300 mmol/L sucrose, 0.5% NP-40 and proteinase inhibitor cocktail (1.0 mmol/L phenylmethylsulfonyl fluoride, 1.0 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 μmol/L pepstatin A, leupeptin 1 μmol/L, 0.1 μmol/L aprotinin) for 3 min on ice and then centrifuged at 7,050g for 20 s. The supernatants were collected as cytosolic protein lysates. The precipitants were resuspended in 20 mmol/L HEPES, pH 7.9, 1.5 mmol/L MgCl2, 240 mmol/L NaCl, 1 mmol/L dithiothreitol, 0.2 mmol/L EDTA, 25% glycerol and proteinase inhibitor cocktail on ice for 30 min and were then centrifuged at 13,000g for 5 min. The supernatants were collected as nuclear protein lysates.

Western Blotting

After treatment for 2 h with or without iloprost (10−7 mol/L), the mDCs were stimulated with 10 μg/mL poly I:C for 1 h and then lysed with equal volumes of ice-cold 150-μL lysis buffer. After centrifugation at 13,000g for 15 min, equal amounts
of cell lysates (20 μg) were analyzed by Western blotting with anti-p65, anti-MAPK (p38, ERK and JNK), anti–phospho-p65 (pp65) and anti–phospho-MAPK (pp38, pERK and pJNK) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). MAPK-p38 activities in cells were measured by nonradioactive MAPK-p38 assay kits (Cell Signaling Technology, Danvers, MA, USA) using the protocols recommended by the manufacturer. Activating transcription factor (ATF)2 was used as substrates for p38 MAPK assay, and phospho-ATF2 and ATF2 were analyzed using horseradish peroxidase–conjugated secondary antibody and the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Sunnyvale, CA, USA).

T-Cell Stimulation Assay

Autologous CD4+ T cells were purified from PBMCs with human CD4 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Isolated CD4+ T cells (10^6/well) were cocultured with iloprost-treated or vehicle-treated mDCs (10^5/well) as described above in 24-well round-bottom plates in 1 mL/well for 5 d in the presence of anti-CD3 and anti-CD28 antibodies (eBioscience, San Diego, CA, USA). In some conditions, CD4+ T cells were cocultured with iloprost-treated mDCs in the presence of anti–IL-10 antibody (0.1 μg/mL; eBioscience). Supernatants were collected for IL-13, IFN-γ and IL-10 measurement.

Flow Cytometry Analysis

Isolated mDCs were cultured in 12-well round-bottom plates (10^5/1 mL/well) and were treated with iloprost (10^-7 mol/L) for 2 h and then stimulated with poly I:C (10 μg/mL) for 48 h and were harvested and washed three times with PBS for direct immunofluorescence staining using fluorescein isothiocyanate–labeled monoclonal antibodies to CD11c, CD40 or CD80, and phycoerythrin–labeled monoclonal antibodies to CD86 or human leukocyte antigens (HLA)-DR. All fluorescence-conjugated monoclonal antibodies were purchased from eBioscience. The surface markers of mDCs were analyzed using a FACSscan flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation (ChIP) assay was performed as described in our previously published work (20,22). Briefly, 5 × 10^5 mDCs in each condition were treated with 1% formaldehyde for 10 min at room temperature. Lysed cells were sonicated and immunoprecipitated overnight at 4°C with anti-trimethylated H3K4 antibody (Upstate Biotechnology, Waltham, MA, USA) or rabbit anti-BSA (Sigma-Aldrich) as a control. Antibody-bound complexes were collected with a slurry of protein A (Invitrogen, Carlsbad, CA, USA) and were washed extensively, and immune complexes were eluted. DNA was extracted by phenol–chloroform after reverse cross-linking for 6 h at 65°C and after protein removal by proteinase K (200 μg/mL; Roche Diagnostics, Nutley, NJ, USA) treatment in the presence of 20 μg/mL glycogen. The DNA was finally RNase treated (40 μg/mL; Roche Diagnostics, Nutley, NJ, USA) for 30 min at
37°C and quantitated before analyses. Total DNA amount of each DNA sample was measured. Equal DNA amount of each sample was used to perform polymerase chain reaction to quantitate the amount of DNA from the promoter and enhancers regions of the TNFA gene encompassing the various TNF-α promoter regions relative to the transcription start sites (17): TNF1 (+99/−42); TNF2 (+32/−119), TNF3 (−100/−250), TNF4 (−195/−345), 1700 (−1694/−1758), +1417 (+1391/+1431) and +720 (+762/+799). Polymerase chain reactions were run on the ABI 7700 Taqman thermocycler (Applied Biosystems, Foster City, CA, USA). All Taqman reagents were purchased from Applied Biosystems. The relative amounts of the amplified product were normalized to the total input DNAs.

Statistical Analysis

For each experiment, three replicates were performed for each subject’s mDCs, and at least three subjects’ mDCs were used to confirm the results presented. All data are presented as the mean ± standard deviation (SD). Differences between experimental and control groups were analyzed using the Wilcoxon signed-rank test. A P value <0.05 was considered indicative of significant between-group differences.

RESULTS

Iloprost and Treprostinil Enhanced IL-10 and Suppressed TNF-α Expression in Human mDCs

To investigate the potential effect of PGI2 analogs on the expression of cytokines in human mDCs, mDCs isolated from healthy subjects were treated with varying doses of iloprost or treprostinil, either alone or in combination with toll-like receptor (TLR)-3 agonist poly I:C. As shown in Figure 1, iloprost (10−9 to 10−7 mol/L) alone or combined with poly I:C could enhance IL-10 expression in human mDCs (Figures 1A, B). Treprostinil (10−8 and 10−7 mol/L) alone could also enhance IL-10 production in human mDCs (Figure 1C). Of interest, iloprost (10−8 to 10−7 mol/L) could suppress poly I:C-induced TNF-α expression (Figure 1D), and treprostinil at a higher concentration (10−6 mol/L) could also suppress poly I:C–induced TNF-α expression (Figure 1E) in human mDCs. However, iloprost or treprostinil alone without the presence of poly I:C had no effect on TNF-α expression (data not shown).

PGL2 Analogs Modulated IL-10 and TNF-α Expression in mDCs via the IP and EP Receptors but Not the PPARs

It is known that PGI2 analogs can exert their function through the IP and EP receptors (24) and are also PPAR ligands with antiinflammatory actions (25). We have previously demonstrated that iloprost and treprostinil modulate chemokine expression partly via the PPARs in human monocytes (20). To examine whether the modulatory effect of PGI2 analogs on IL-10 and TNF-α expression is mediated through the IP receptors, EP receptors or PPARs, mDCs were pretreated with the IP receptor antagonist (CAY 10449), the EP receptor antagonists (SC 19220 as EP1 receptor antagonist; AH6809 as EP2 receptor antagonist; GW627368X as EP4 receptor antagonist) or PPAR antagonists (GW6741 as PPAR-α antagonist; GW9662 as PPAR-γ antagonists) at the concentration of 10−6 to 10−5 mol/L. As shown in Figure 2, the addition of IP receptor antagonist CAY 10449 (10−6 to 10−5 mol/L) reversed iloprost-enhanced IL-10 expression (Figure 2A). The EP1 receptor antagonist SC 19220 (10−5 mol/L), but not EP2 or EP4 receptor antagonists, reversed iloprost-enhanced IL-10 expression (Figure 2B). These data suggested that ilo-
The suppressive effect of iloprost (C) or treprostinil (D) on poly I:C–induced TNF-α expression was abrogated by the treatment of iloprost or treprostinil. Neither PPAR-α antagonist nor PPAR-γ agonist changed the enhancing effect of iloprost (A) or treprostinil (B) on IL-10 expression, and the suppressive effect of iloprost (C) or treprostinil (D) on poly I:C–induced TNF-α expression. Results represented are the mean ± SD of three independent experiments using mDCs from three subjects.

Figure 4. The modulatory effects of PGI2 analogs on IL-10 and TNF-α expression is independent of PPARs in human mDCs. To investigate the involvement of PPARs in the modulatory effect of PGI2 analogs on IL-10 and TNF-α expression in human mDCs, the cells were pretreated with PPAR-α antagonist (GW 6741) and PPAR-γ agonists (GW9662) 1 h before the treatment of iloprost or treprostinil. Neither PPAR-α antagonist nor PPAR-γ agonist changed the enhancing effect of iloprost (A) or treprostinil (B) on IL-10 expression, and the suppressive effect of iloprost (C) or treprostinil (D) on poly I:C–induced TNF-α expression. Results represented are the mean ± SD of three independent experiments using mDCs from three subjects.

Prostaglandin I2 (PGI2) analogs, such as iloprost and treprostinil, are known to modulate cytokine production in immune cells. PGI2 analogs can increase the expression of IL-10, a cytokine that suppresses inflammation, and decrease the expression of TNF-α, a pro-inflammatory cytokine. To investigate the role of PGI2 analogs in the modulation of IL-10 and TNF-α expression, we measured the effects of PGI2 analogs on IL-10 and TNF-α expression in human mDCs. We found that PGI2 analogs modulated IL-10 and TNF-α expression through the cAMP and Ca2+ pathways.

**PGI2 Analogs Modulated IL-10 and TNF-α Expression in mDCs via the cAMP Pathway, and Iloprost Also Modulated IL-10 and TNF-α Expression via the Ca2+ Pathway**

IP and EP receptors are G protein–coupled receptors, and the cellular responses are based on the types of G protein. It is known that IP, EP2 or EP4 receptors activate the G protein Gs, which leads to an increase of intracellular cAMP (20,24). We next examined whether iloprost or treprostinil could elevate intracellular cAMP in human mDCs. As shown in Figures 4A and B, iloprost (10⁻⁸ to 10⁻⁷ mol/L) and treprostinil (10⁻⁷ mol/L) result in an increase of intracellular cAMP in mDCs. Next we used forskolin, an adenyl cyclase activator, to examine whether elevating cAMP could confer a similar effect in human mDCs. Forskolin also enhanced IL-10 expression in human mDCs. These data suggested the modulatory effects of PGI2 analogs on IL-10 and TNF-α expression were through the IP/EP2/EP4→cAMP pathway.

The activation of the EP1 receptor by ligands increases the level of the intracellular Ca²⁺, which regulates the downstream signals (26). Because the involvement of EP1 receptor in the modulatory effects of iloprost on IL-10– and poly I:C–induced TNF-α expression is suggested by the results using EP1 receptor antagonist, we next investigated whether the EP1-Ca²⁺ pathway is involved in the modulatory effects of iloprost. We used flow cytometry to investigate the effect of iloprost on the change of intracellular Ca²⁺ levels. As shown in Figures 4E and F, treatment with iloprost (10⁻⁷ mol/L) enhanced the fluorescence intensity of Fluo-3-AM, and the effects could be partly reversed by EP1 receptor antagonist SC 19220 (10⁻⁵ mol/L), suggesting that iloprost increased intracellular Ca²⁺ levels via the EP1 receptor. BAPTA-AM, the intracellular Ca²⁺ chelator, partly abrogated the modulatory effects of iloprost on IL-10–induced (Figure 5G) and poly I:C–induced TNF-α expression.
Taken together, this evidence suggested that iloprost may also partly modulate IL-10- and poly I:C-induced TNF-α expression via the EP1 receptor–Ca2+ pathway.

**Iloprost Suppressed Poly I:C–Induced TNF-α Expression via the MAPK-p38-ATF2 Pathway**

It is known that the activation of Toll-like receptors on DCs can lead to activation of the MAPK pathways including the MAPK-p38, JNK, and ERK pathways (27). It is also known that the nuclear factor (NF)-κB pathway is involved in the expression of proinflammatory cytokines in DCs (14). We next investigated whether the effects of PGI2 analogs on TNF-α expression is via these pathways. As shown in Figure 5, all three MAPK inhibitors suppressed poly I:C–induced TNF-α expression (Figure 6A), suggesting that all three MAPK pathways are involved in poly I:C–induced TNF-α expression in mDCs. Western blotting revealed that iloprost suppressed poly I:C–induced phospho-p38 and phospho-ATF2 expression in mDCs (Figures 6B, C). However, iloprost had no effect on poly I:C–induced phospho-p65, phospho-ERK or phospho-JNK expression in mDCs (data not shown). These data suggested that iloprost may suppress poly I:C–induced TNF-α expression in human mDCs via the MAPK-p38-ATF2 pathway.

**Iloprost Suppressed Poly I:C–Induced TNF-α Expression via Histone Trimethylation**

TNF-α expression can be regulated epigenetically via core histone modification in monocytes and macrophages (17). To investigate whether the effect of iloprost on poly I:C–induced TNF-α expression in mDCs is via epigenetic regulation, ChIP assays were performed with antibodies recognizing trimethylated H3K4 as a marker of gene activation (28). As shown in Figure 7, iloprost downregulated poly I:C–induced H3K4 trimethylolation in the TNFA gene promoter region from upstream to downstream of the transcription start site (Figure 7A). Be-
EFFECT OF PGI₂ ANALOGS ON HUMAN MYELOID DCS

cause histone trimethylation is mediated by methyltransferase, we next examine whether the downregulation of H3K4 trimethylation by iloprost is associated with the downregulation of the H3K4-specific methyltransferases and MLL and WDR5 proteins (9). Intriguingly, poly I:C-induced translocation of MLL and WDR5 protein from cytoplasm into nucleus, and iloprost, can partly reversed the translocation of MLL and WDR5 proteins from cytoplasm to nucleus by poly I:C (Figures 7B, D). In the present study, we show that iloprost inhibited the poly I:C-induced phosphorylation of MAPK-p38. Therefore, we next investigated whether MAPK-p38 signaling is involved in poly I:C-induced subcellular localization of MLL and WDR5. As shown in Figure 7C, the MAPK-p38 inhibitor SB203580 also suppressed translocation of MLL and WDR5 proteins from cytoplasm to nucleus. The evidence suggested that the inhibitory effect of iloprost on the poly I:C-induced translocation of MLL and WDR5 from cytoplasm to nucleus may be, at least partly, via the MAPK-p38 pathway. Taken together, the present study suggested that the effect of iloprost on histone H3K4 trimethylation in the TNFA gene promoter region may play an important role in regulating the expression of TNF-α in human mDCs.

Iloprost Had No Effect of CD86, CD80, CD40 and HLA-DR Expression on mDCs

To examine the effect of iloprost on poly I:C-induced mDC maturation, the expression of costimulatory molecules including CD86, CD80, CD40 and HLA-DR was investigated by flow cytometry. However, during the culture time period, there was no significant difference in the expression levels of DC maturation markers, including CD86, CD80, CD40 and HLA-DR, as judged by flow cytometry (data not shown).

Iloprost-Treated mDCs Suppressed IL-13, IFN-γ and IL-10 Production in CD4⁺ T Cells

To investigate the effect of iloprost on the ability of mDCs to stimulate T-cell response, human mDCs were treated with varying doses of iloprost (10⁻⁷ to 10⁻³ mol/L) and were cocultured with autologous CD4⁺ T cells at a ratio of 1:10 (10⁵ mDCs/10⁴ CD4⁺ T cells) in the presence of anti-CD3 and anti-CD28. In some cases, IL-10 neutralizing antibody was used to verify whether the effects of iloprost on IL-13/IFN-γ production by T cells is through IL-10 regulation. As shown in Figure 8, IL-13 (Figure 8A), IFN-γ (Figure 8B) and IL-10 (Figure 8C) production by CD4⁺ T cells was significantly suppressed by iloprost-treated mDCs. The presence of IL-10-neutralizing antibody did not change the effect of iloprost-treated mDCs on the IL-13 and IFN-γ production by T cells (Figure 8D). These data suggested iloprost-treated mDCs may attenuate the ability of mDC to stimulate T-cell response, and the effects were not indirectly mediated by IL-10.

DISCUSSION

DCs are the chief orchestrators of immune responses. The crucial task of mDCs is the continuous surveillance of antigen-exposed sites throughout the body and the initiation of primary T-cell responses including T-cell polarization into Th1 and Th2 cells by secreting cytokines and expressing costimulatory molecules after activation (17,29). DCs, particular in mDCs, have a specific and important role in pathogenesis of human asthma (11–13). Recently, PGI₂ is regarded as a potential treatment of asthma by their antiinflammatory effect in vitro (14,30) and in animal model (15). The antiinflammatory effect of PGI₂ analogs by altering the function of DCs has been revealed by our previous work using human plasmacytoid DCs (16) and by the work of Muller et al. (31) using human monocyte-derived DCs (31). However, the effect of PGI₂ analogs on human mDCs is not elucidated. In the present study, we demonstrated, for the first time, the effect of PGI₂ analogs (iloprost and treprostinil) on human mDCs and found that although iloprost had no effect on costimulatory molecules expression, iloprost and treprostinil could enhance IL-10 and suppress poly I:C-induced TNF-α expression in mDCs. In addition, iloprost could suppress the ability of mDCs to stimulate Th1 (IFN-γ) and Th2 (IL-13) response. These all implicate the potential role of PGI₂ analogs in treating asthma by altering the function of mDCs.
Prostaglandins are derived from arachidonic acid by stepwise conversion and are important endogenous inflammatory mediators, controlling immune stimulation and inflammation by the effects of prostaglandins on cytokine production (32). IP receptor activation by PGI2 or its analogs can result in vasodilation and antithrombotic and antiinflammatory effects (14,33). PGI2 analogs are available in different formulations and are potent ligands with different binding affinities for the various prostanoid receptors. Iloprost was reported with affinity to the IP, EP1, EP2, EP3 and EP4 receptors, and treprostinil was reported with affinity to the IP and EP2 receptors (34–36). The physiological activities of the analogs depend on the receptor they activate, and these effects can be receptor-specific. For example, while treprostinil is clearly a potent IP receptor agonist (37), the effect of treprostinil on inhibiting phagocytosis, bacterial killing, and cytokine generation in the alveolar macrophage is via the EP2 receptor but not IP receptor (24). In the present study, we demonstrate that the modulatory effects of PGI2 analogs on IL-10 and TNF-α expression in human mDCs involved different types of receptors. To enhance IL-10 expression, iloprost acted through the IP and EP1 receptor, whereas treprostinil acted through the EP4 receptor only. To suppress poly I:C–induced TNF-α expression, iloprost acted through IP, EP1, EP2 and EP4 receptors, while treprostinil acted through IP receptor only. The different involvement of IP/EP receptors of iloprost and treprostinil may partly explain their different potency in modulating cytokine expression. Although PGI2 analogs are PPAR ligands with antiinflammatory actions (25) and our previous work demonstrated that iloprost and treprostinil modulate chemokines expression partly via PPARs in human monocytes (20), the present study revealed that the PPARs were not involved in the modulatory effect of PGI2 analogs on IL-10 and TNF-α expression in human mDCs.

In the present study, we also demonstrated the responsible signal transduction pathway that was activated by the prostanooid receptor for the modulatory effect of PGI2 analogs on IL-10– and poly I:C–induced TNF-α expression in human mDCs. It has been suggested that the signaling followed by the activation of prostanoid receptor depends on the types of coupled G protein. The activation of the G protein may vary from ligands and the ligand concentration and finally evokes different cellular responses (34). The IP and EP2/EP4 receptors are coupled to Gs protein and activates adenyl cyclase, which results in a burst of intracellular cAMP, whereas EP1 receptors are coupled to the Gq protein and mediate the increase of intracellular Ca2+ (38). In the present study, we

Figure 7. Iloprost suppressed poly I:C–induced TNF-α expression in mDCs via histone H3K4 trimethylation in the TNFA gene promoter region. Isolated mDCs were pretreated with iloprost for 2 h and then stimulated with poly I:C for 1 h and were used for ChIP assay using anti-H3K4 antibodies. The cells were also used for cytosolic and nuclear protein analysis by Western blotting using anti-MLL and anti-WDR5 antibodies. The nuclear and cytosolic fractionation technique was verified using anti-α-tubulin and anti-histone H3 antibodies. (A) Iloprost downregulated poly I:C–induced H3K4 trimethylation in the TNFA gene promoter region. (B) Iloprost suppressed poly I:C–induced translocation of cytosolic H3K4–specific methyltransferases MLL and WDR5 proteins into nucleus. The nuclear fraction is lack of α-tubulin, and the cytosolic fraction is lack of H3. To investigate whether MAPK-p38 signaling is responsible for the translocation of MLL and WDR5 from cytoplasm to nucleus, mDCs were pretreated with MAPK-p38 inhibitor SB203580 for 2 h and then stimulated with poly I:C for 1 h. (C) SB203580 suppressed poly I:C–induced translocation of MLL and WDR5 from cytoplasm to nucleus. (D) Densitometry analysis of the Western blot data shown in (B). Results represented are the mean ± SD of three independent experiments using mDCs from three subjects for the ChIP assay and densitometry analysis. For Western blotting analyses, one experiment representative of three is shown. *P ≤ 0.05 compared with poly I:C–treated cells (A and D). OD, optical density. A: Control; I: poly I:C; B: iloprost + poly I:C.
EFFECT OF PGI₂ ANALOGS ON HUMAN MYELOID DCS

Figure 8. Iloprost-treated mDCs suppressed IL-13, IFN-γ and IL-10 production by CD4+ T cells. Autologous CD4+ T cells were isolated and then cocultured with vehicle-treated or iloprost-treated mDCs (10⁵ mDCs/10⁶ T cells) for 5 d in the presence of anti-CD3 and anti-CD28 antibodies with or without the addition of IL-10 neutralizing antibody. Supernatants were collected for IL-13, IFN-γ and IL-10 measurement. Iloprost-treated mDCs significantly suppressed IL-13 (A), IFN-γ (B) and IL-10 production (C) by CD4+ T cells. (D) The addition of IL-10 neutralizing antibody did not change the effect of iloprost-treated mDCs on IL-13 and IFN-γ production by T cells. Results represent the mean ± SD of six independent experiments using mDCs from six subjects in the experiments without the addition of IL-10 neutralizing antibody and of three independent experiments using mDCs from three subjects in the experiments with the addition of IL-10 neutralizing antibody. *P ≤ 0.05 compared with the group of vesicle-treated mDC/T-cell coculture. §P ≤ 0.05 compared with the group of iloprost-treated mDC/T-cell coculture without the addition of IL-10 neutralizing antibody. (D) ■ Control; □ iloprost (10⁻⁷ mol/L); ■ iloprost (10⁻⁷ mol/L) + anti-IL-10.

Demonstrated that iloprost, and treprostinil at higher concentrations, increased intracellular cAMP levels. Forskolin, the adenyl cyclase activator, modulated similar effects on IL-10 and TNF-α expression. These results suggested the enhancing effect on IL-10 may be via the IP-cAMP pathway by iloprost and via the EP4-cAMP pathway by treprostinil, and the suppressive effect on poly I:C-induced TNF-α may be via the IP-EP2/EP4-cAMP pathway by iloprost and via the IP-cAMP by treprostinil. Interestingly, the potency of forskolin on enhancing IL-10 and suppressing poly I:C-induced TNF-α expression is quite different. Compared to the nearly complete reversing effect on iloprost-enhanced IL-10 expression, the IP receptor antagonist CAY 10449 restored the suppressive effect of iloprost on poly I:C-induced TNF-α expression to a less extent. These observations implicate there may be a cAMP-independent pathway that mediated the suppressive effect of iloprost on poly I:C-induced TNF-α expression. We found that iloprost increased intracellular Ca²⁺ levels via the EP1 receptor, and the intracellular Ca²⁺ chelator BAPTA-AM abrogated the modulatory effect of iloprost on IL-10- and poly I:C-induced TNF-α expression. The evidence indicates that in addition to IP-EP-cAMP pathways, iloprost can also modulate IL-10- and poly I:C-induced TNF-α expression via the EP1-Ca²⁺ pathway. The higher potency on increasing intracellular cAMP level and the activation of an additional pathway may partly explain the higher potency of iloprost in modulating cytokine expression compared to that of treprostinil. Our results may offer an experimental basis for investigating the different functions and efficacy between various PGI₂ analogs for clinical application.

The MAPK pathways are fundamental regulators for chemoattraction, inflammatory mediator production and activation in immune cells in response to stimulation by TLR agonists (27). In asthmatic patients, the level of phosphorylation of p38 and ERK is positively correlated with disease severity (39). In the present study, we used the MAPK-p38 inhibitor (SB203580) and Western blotting to verify that iloprost may suppress poly I:C-induced TNF-α expression via, at least partly, the MAPK-p38-ATF2 pathway, providing further understanding for the intracellular mechanism of PGI₂ analogs on cytokine modulation.

In the present study, we furthermore provided an important novel finding for the epigenetic regulation of iloprost on TNF-α expression in human mDCs. Histone and DNA modifications are associated with gene transcription. For example, acetylation of core histone by histone acetyltransferase allows the chromatin structure to transform from the resting closed conformation to an activated open form, leading to gene expression. Asthma is associated with overexpression of inflammatory genes in the airway. It has been shown that the activity of histone acetyltransferase is increased in bronchial biopsy and alveolar macrophages isolated from asthmatic patients (40), and the proinflammatory cytokine, TNF-α, can be regulated epigenetically with histone acetylation in monocytes and macrophages (17). In addition to histone acetylation, histone methylation is also associated with either positive or negative transcriptional states, depending on the sites of modification. In our recently published work, we demonstrated that iloprost can modulate Th1- and Th2-related chemokine
expression via histone acetylation and trimethylation in human monocytes (20). In the present study, we revealed, for the first time in human mDCs, that poly I:C could induce histone H3K4 trimethylation in the TNFA gene promoter region, and iloprost could downregulate poly I:C-induced H3K4 trimethylation in the TNFA gene promoter region. We also verified that the suppressive effect of iloprost on poly I:C-induced H3K4 trimethylation was produced by inhibiting the poly I:C-induced translocation of H3K4-specific methyltransferases MLL and WDR5 proteins from cytoplasm into nucleus. To our best knowledge, this novel mechanism by which iloprost modulates TNF-α expression in the present study is reported in the literature.

Interestingly, phosphorylation signaling has been shown to play an essential role in regulating the function and interaction between proteins, maintaining the stability and participating in the localization of the protein. Recently, it was suggested that the phosphorylation modification on Thr-912 residue of MLL protein controls its subcellular localization and is required for mitotic entry (41). In the present study, we showed that iloprost suppressed poly I:C-induced phosphorylation of MAPK-p38, and by using the MAPK-p38 inhibitor SB203580, we also showed that the poly I:C-induced translocation of MLL and WDR5 proteins from cytoplasm to nucleus was MAPK-p38 dependent. These results suggest that iloprost may suppress poly I:C-induced translocation of MLL and WDR5 proteins via, at least partly, the MAPK-p38 pathway. Taken together, our findings suggest the importance of epigenetic regulation by which PGI₂ analogs exert their antiinflammatory functions.

CONCLUSION

In conclusion, the present study provided the evidence for the effects of PGI₂ analogs on human mDCs. Our study suggested that PGI₂ analogs may induce tolerogenic function of human mDCs by modulating cytokine production (enhancing antiinflammatory cytokine IL-10 and suppressing proinflammatory cytokine TNF-α) and by inhibiting the ability of mDCs for T-cell stimulation. The suppressive effect of iloprost on TNF-α expression was via the IP/EP2/EP4-cAMP and EP1 receptor–Ca²⁺ pathway, the MAPK-p38-ATF2 pathway and epigenetic regulation by histone modification with downregulation of H3K4 trimethylation via inhibiting the translocation of H3K4-specific methyltransferases MLL and WDR5 proteins. Because of the key roles of mDCs in pathogenesis of human asthma, our results supported current evidence for the potentiality of PGI₂ analogs as asthma treatment.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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