

Dendritic cells treated with a prostaglandin I₂ analog, iloprost, promote antigen-specific regulatory T cell differentiation in mice

Tzu-Hsuan Wong^{a,b}, Rung-Jiun Gau^c, Yu-Fang Chen^a, Hsin-Hsin Shen^c, Carl Tsai-Yu Lin^c, Sen-Lu Chen^c, Jau-Ling Suen^{a,b,d,*}

^a Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC

^b Research Center for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC

^c Biomedical Technology and Device Research Laboratories, Industrial Technology Research Institute, Hsinchu, Taiwan, ROC

^d Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, ROC

ARTICLE INFO

Keywords:

Allergic inflammation
prostaglandin I₂
mPEG-PLGA hydrogel
Regulatory T cell
Tolerogenic dendritic cell
PD-L1

ABSTRACT

Iloprost, a stable prostaglandin I₂ (PGI₂) analog, can inhibit allergic inflammation in an ovalbumin (OVA)-induced asthma model via inhibition of airway dendritic cell (DC) function. However, the underlying mechanism of PGI₂ signaling-mediated immunosuppression remains unclear. This study explored whether iloprost-treated DCs can suppress inflammation by promoting antigen-specific regulatory T cell (Treg) differentiation through PGI₂-G-protein-coupled receptor (IP). We established an allergic lung inflammation model using a hydrogel biomaterial delivery system and observed that iloprost significantly suppressed OVA-induced Th2 lung inflammation and increased the frequency of OVA-specific Tregs *in vivo*. We further observed that iloprost-treated DCs displayed tolerogenic characteristics, including low inflammatory cytokine (IL-12, TNF- α , IL-6, IL-23) expression levels, high anti-inflammatory cytokine (IL-10) production, and a semimature phenotype. In addition, iloprost-treated DCs increased OVA-specific CD4⁺Foxp3⁺ T cell differentiation from naïve T cells in an IP-dependent pathway *in vitro* and *in vivo*. Blocking experiments showed that iloprost-treated DCs promoted Treg differentiation, at least in part, through programmed death ligand 1 (PD-L1), whereas iloprost-induced PD-L1 expression in DCs was through the IP receptor. Furthermore, iloprost treatment suppressed DC-mediated airway inflammation and increased the frequency of OVA-specific Tregs through PD-L1 *in vivo*. Taken together, these results show that PGI₂-IP signaling mediated by iloprost in DCs may lead to immune tolerance, suggesting that the PGI₂ analog has the potential to be applied therapeutically for tolerogenic DC immunotherapy in autoimmune diseases or allergic asthma.

1. Introduction

Dendritic cells play a key role in regulating immune responses, not only initiating adaptive immunity but also maintaining immune tolerance [1,2]. Several modulators have been developed to generate tolerogenic dendritic cells (TDCs) *ex vivo*, and the number of clinical trials for TDC-based therapies for various inflammatory diseases has also grown vigorously in recent years [3,4]. The advantage of TDC-based therapy is that it can simultaneously induce tolerance to multiple T-cell epitopes due to the excellent antigen (Ag)-presenting ability of DCs [3,4]. Therefore, finding new possibilities from current clinical drugs could be a time-saving and highly safe solution to develop TDC-based immunotherapies.

Iloprost, a prostaglandin I₂ (PGI₂) analog used in pulmonary arterial

hypertension (PAH) treatment [5], has been reported to have anti-inflammatory effects by suppressing DC function in a mouse asthma model [6], suggesting its potential application in TDC-based immunotherapy. PGI₂ is an important lipid mediator of prostaglandins (PGs), which are generated by a stepwise conversion of arachidonic acid [7]. PGI₂ has been shown to have anti-inflammatory function in a murine respiratory syncytial virus (RSV) infection model [8] and ovalbumin (OVA)-induced allergy model [9–11]. Previous studies have shown that PGI₂-G-protein-coupled receptor (IP) signaling may mediate the anti-inflammatory effect by modulating the function of bone marrow-derived dendritic cells (BM-DCs) *in vitro* [12–14]. In addition, PGI₂ receptor-deficient mice displayed increased allergic inflammation to OVA challenge compared with wild-type mice [15,16]. These studies suggest that PGI₂ signaling may induce TDC differentiation to mediate

* Corresponding author at: No. 100, Shin-Chuan 1st Road, Sanmin Dist., Kaohsiung City 80708, Taiwan, ROC.

E-mail address: jlsuen@kmu.edu.tw (J.-L. Suen).

<https://doi.org/10.1016/j.intimp.2019.106106>

Received 29 July 2019; Received in revised form 17 November 2019; Accepted 29 November 2019

1567-5769/© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Abbreviations

Ag	antigen
BALF	bronchoalveolar lavage fluid
BM-DCs	bone marrow-derived dendritic cells
CFSE	carboxy-fluorescein diacetate succinimidyl ester
ELISA	enzyme-linked immunosorbent assay
ICOS-L	inducible costimulator-ligand
IP	PGI ₂ -G-protein-coupled receptor

mPEG-PLGA	methoxy polyethylene glycol-co-poly(lactic-co-glycolic acid)
OVA	ovalbumin
PAH	pulmonary arterial hypertension
PD-L1	programmed death ligand 1
PGI ₂	prostaglandin I ₂
TDC	tolerogenic dendritic cell
Treg	regulatory T cell

the immunosuppressive effect. However, the precise mechanism involved in the anti-inflammatory effect mediated by PGI₂ has not been elucidated.

Regulatory T cells (Tregs) play an important role in maintaining homeostasis via their antigen-specific immunosuppressive function. Adaptive Tregs can be generated through interactions with TDCs and mediate immunosuppression in various inflammatory diseases [17]. Many studies have shown that TDCs induce Ag-specific tolerance by several mechanisms including promotion of adaptive Treg conversion from naïve T cells, expansion of preexisting Tregs, release of IL-10, and expression of indoleamine 2,3-dioxygenase (IDO) [18–21]. Additionally, various soluble or membrane-bound molecules on DCs are also likely to be involved in Treg induction [22], including programmed death ligand 1 (PD-L1), ILT3/ILT4, SLAM, inducible costimulator (ICOS) and glucocorticoid-induced TNFR-related protein (GITR) [23–27]. Therefore, understanding the precise mechanisms involved in Treg differentiation induced by TDCs *in vivo* will facilitate the development of cell-based tolerogenic immunotherapies.

In our study, we demonstrated that iloprost-treated DCs promoted Ag-specific Foxp3⁺ Treg differentiation, at least in part, through PD-L1 *in vitro* and *in vivo*. As iloprost has been used in clinics for PAH treatment [28], iloprost-based TDC immunotherapies could be developed for various inflammatory diseases.

2. Materials and methods

2.1. Mice

All animal experiments were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of the Kaohsiung Medical University (IACUC: 101048). Female BALB/cByJNarl and DO11.10 mice, aged 5–8 weeks, were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Female C.Cg-Foxp3^{tm2Tch}/J (Foxp3^{EGFP}) mice and Thy1.1 mice were purchased from the Jackson Lab (Bar Harbor, ME, USA). All mice were maintained in a specific pathogen-free facility by the institution's animal center.

2.2. Establishment and assessment of allergic lung inflammation models

For the OVA-induced asthma model (Fig. 1), BALB/c mice were subcutaneously injected with a mixture containing 15% methoxy polyethylene glycol-co-poly(lactic-co-glycolic acid) (mPEG-PLGA) hydrogel (from Industrial Technology Research Institute, Hsinchu, Taiwan), iloprost (5 µg/mouse; Cayman Chemical, Ann Arbor, MI, USA), and OVA (0.2 µg/mouse; grade V; Sigma-Aldrich, St. Louis, MO, USA) on day 0 and day 21. On days 26–28, mice were challenged with 3% OVA aerosol to induce lung inflammation and sacrificed on day 29.

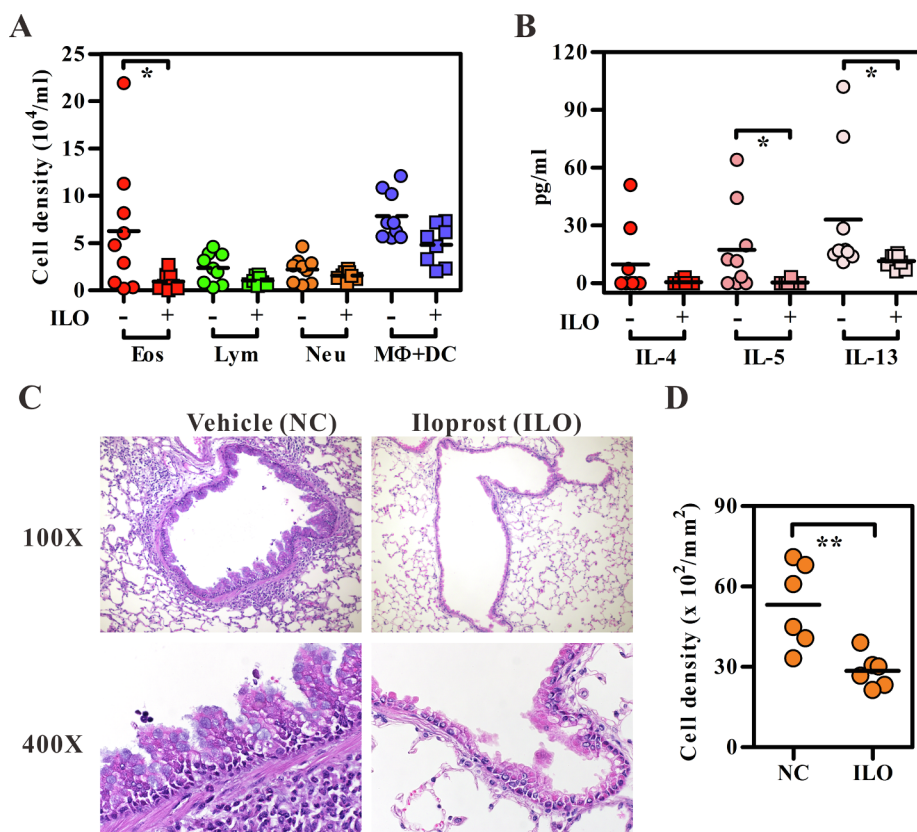


Fig. 1. The anti-inflammatory effect of iloprost on allergic lung inflammation *in vivo*. BALB/c mice were subcutaneously administered an mPEG-PLGA hydrogel mixture containing OVA/iloprost (ILO) or OVA/vehicle (NC) on day 0 and day 14. After 4 days, the mice received three daily OVA aerosol challenges. Cellular composition (A) and cytokine pattern (B) in BALFs were analyzed using flow cytometry and ELISA methods, respectively. N = 8–9 mice per group. (C) Representative lung sections stained with hematoxylin and eosin are selected from six mice in each group. Data are representative of two independent experiments. (D) The density of hematoxylin⁺ cells in lung sections. The line within the vertical points marks the mean for each group. Eos: eosinophils; Lym: lymphocytes; Neu: neutrophils; MΦ: macrophages; DC: dendritic cells. *p < 0.05 or **p < 0.01 compared with vehicle. The cell density of each subset or cytokine level in BALFs in the mice without OVA sensitization was less than 0.1 × 10⁴ cells/ml or undetectable, respectively.

The vehicle for iloprost was 0.14% methyl acetate.

Temperature-sensitive hydrogel is a polylactic acid-glycolic acid ring-opening polymerization, and stannous octoate was used as a catalyst. Methoxypolyethylene glycol was used as an initiator to synthesize methoxypolyethylene glycol/polylactic acid-glycolic acid double-coupling copolymer. The synthesis of mPEG-PLGA hydrogel was described in a previous study [29].

The BM-DC-induced asthma model (Fig. 7) was established according to a previous study [30]. In brief, the BM-DCs (1×10^6 cells in 40 μ l PBS) treated as described below were intratracheally transferred into syngeneic naive mice. After 10 days, the recipients were challenged daily with 3% OVA aerosol for 15 min for 4 consecutive days. On the day after the last challenge, bronchoalveolar lavage fluid (BALF) cells were collected and stained with PE-Cy7-anti-CD11c (N418; eBioscience, Ireland, UK), FITC-anti-I-A^d/I-E^d (M5/114.15.2; eBioscience), PE-anti-CCR3 (83101; R&D Systems, Minneapolis, MN, USA), APC-anti-CD3 (145-2C11; BD Biosciences, San Diego, CA, USA) and APC-anti-B220 (RA3-6B2; eBioscience) mAbs. The cellular composition of BALF cells was determined by flow cytometry (LSR II; BD Biosciences). Cytokine levels in BALFs were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience).

2.3. Histological analysis of lung pathology

Lung tissue was fixed in formaldehyde (3.7%) and embedded in paraffin. The sections of tissue (3 μ m) were stained with hematoxylin and eosin according to the manufacturer's protocol (NovoLink™, Polymer Detection System, Leica, UK). Images of the stained tissue were captured by a TissueFAXS microscope and the quantification of cell density was processed using HistoQuest. Hematoxylin staining was used as a master marker for cell identification on the basis of nuclear detection. Furthermore, the average nuclear size, discrimination area,

discrimination grey, and background threshold for the master marker was specified. The range of intensities of the master marker (hematoxylin) was set by auto-detection with the software. The number of hematoxylin⁺ cells or area in each defined regions of interests per lung section was analyzed with TissueQuest software. Lung inflammation was evaluated based on the number of hematoxylin⁺ cells per square millimeter.

2.4. BM-DC generation and treatment

Bone marrow cells were cultured with recombinant murine GM-CSF (125 U/ml, Pepro Tech Inc., Rocky Hill, NJ) and 2-mercaptoethanol (50 μ M) for 8 days. Day-8 BM-DCs were treated with iloprost and LPS (1 μ g/ml, *Escherichia coli* O127:B8; Sigma-Aldrich) for an additional 24 h. CAY10441 (1 μ M, Cayman Chemical, Ann Arbor, MI, USA), a specific IP antagonist, was added into day-8 BM-DCs 30 min before iloprost treatment. The cells were harvested for phenotypic analysis and the analysis of T-cell responses, and supernatants were collected for cytokine determination by ELISA (eBioscience). The BM-DC surface markers were analyzed by flow cytometry (LSR II, BD Biosciences) for the expression of CD11c (G418), MHC class II (M5/114.15.2), CD40 (1C10), CD80 (16-10A1), CD86 (GL1), DO11.10 TCR (KJ126), PD-L1 (MIH5), PD-L2 (TY25), SLAM (9D1) and inducible costimulator-ligand (ICOS-L) (HK5.3).

For the BM-DC-induced asthma model (Fig. 7), day-8 BM-DCs were treated with iloprost (40 nM) and OVA (125 μ g/ml) for 6 h and then LPS for an additional 18 h. The harvested BM-DCs were incubated with an anti-PD-L1 blocking mAb (25 μ g/ml, MIH5, eBioscience) at 4 °C for 20 min prior to intratracheal injection into the BM-DC-induced asthma model.

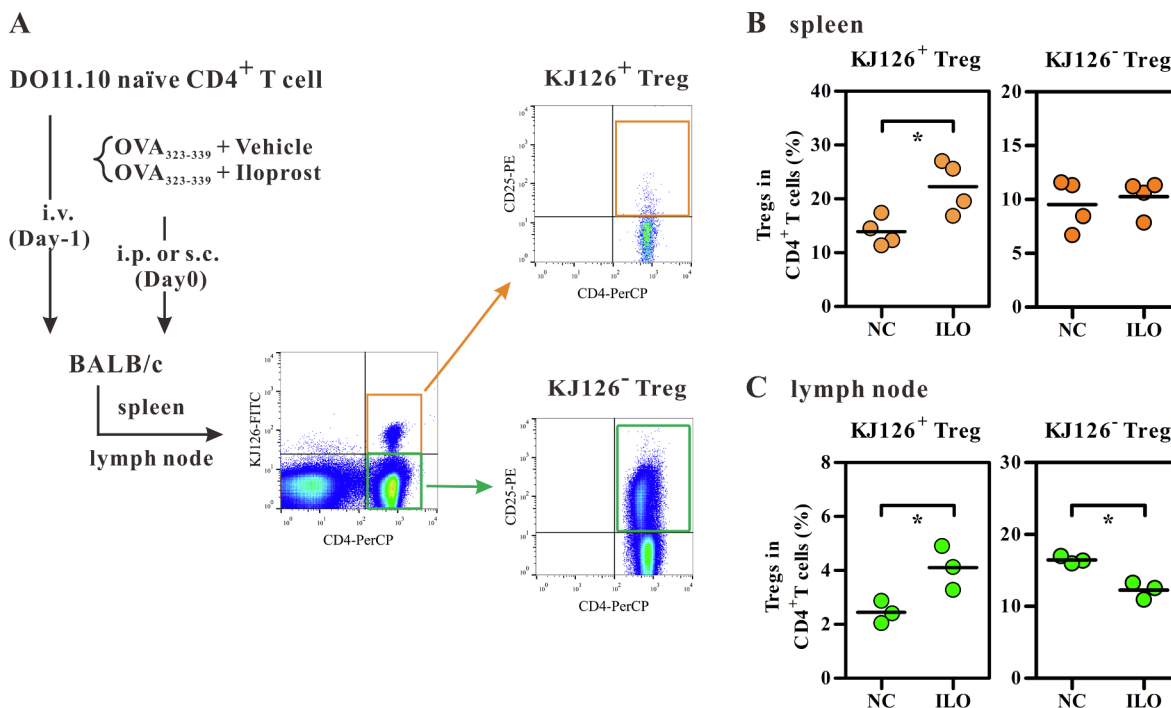


Fig. 2. Ag-specific adaptive Treg differentiation induced by iloprost *in vivo*. Naïve CD4⁺ T cells from DO11.10 mice were intravenously (i.v.) transferred into BALB/c mice, followed by intraperitoneal (i.p.) or subcutaneous (s.c.) administration of the hydrogel mixture containing OVA₃₂₃₋₃₃₉/iloprost or OVA₃₂₃₋₃₃₉/vehicle. After 7 days, splenocytes or lymph node cells were stained with fluorochrome-labeled anti-CD3, CD4, CD25, and KJ126 clonotypic Abs and analyzed using LSR II. (A) Representative dot plots show the expression of CD25 on gated KJ126⁺ (upper panel) or KJ126⁻ (lower panel) CD4⁺ T cells. The results are shown as percentages of KJ126⁺CD4⁺CD25⁺ Tregs or KJ126⁻CD4⁺CD25⁺ Tregs in splenocytes (B) or lymph node cells (C) from intraperitoneally or subcutaneously treated mice, respectively. Symbols represent the frequencies of Tregs in each individual mouse. The line within the vertical points marks the mean for each group. One representative dataset was obtained from two independent experiments. NC: vehicle; ILO: iloprost. **p* < 0.05 compared with vehicle.

2.5. Naïve CD4⁺ T cell and Treg cell purification

For naïve T cell purification, splenocytes were stained with B220-PE (RA3-6B2), CD8α-PE (53–6.7), γ/δ TCR-PE (GL3), CD11b-PE (D12), CD49b-PE (DX5), CD25-PE (PC61), CD19-PE (4G7) and further labeled with anti-PE-microbeads (Miltenyi Biotec, Sunnyvale, CA, USA) for negative selection according to the manufacturer's instructions (autoMACS™ Separator, Miltenyi Biotec, Bergisch Gladbach, Germany). Negatively selected cells were stained with CD62L-PE (MEL-14) and anti-PE-microbeads for further positive selection. Treg cells were purified from splenocytes using a mouse CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec) and sorted by an autoMACS™ Separator according to the manufacturer's instructions. The purities of naïve CD4⁺ T cells and Tregs were at least 92% when analyzed by flow cytometry.

2.6. Adaptive Treg differentiation assay *in vitro* and *in vivo*

For *in vitro* Treg differentiation, 1×10^5 treated BM-DCs from BALB/c mice were cocultured with naïve CD4⁺ T cells from syngeneic Foxp3^{EGFP} or BALB/c mice in RPMI1640-10% FBS with anti-CD3 mAb (1 µg/ml, BD Biosciences), rmTGF-β (60 U/ml; PeproTech), rhIL-2 (100 U/ml; Pepro Tech) and in the presence or absence of CAY10441 (1 µM; Fig. 3C) or blocking mAbs (isotype control or anti-PD-L1; Fig. 6F) at a DC/T ratio of 1: 10 for three days. The EGFP (Foxp3)⁺ CD4⁺ T cells or Foxp3⁺CD4⁺ T cells were analyzed by flow cytometry (LSR II; BD Biosciences).

For *in vivo* Treg differentiation induced by iloprost (Fig. 2), naïve CD4⁺ T cells (2×10^6 cells) from DO11.10 mice were adoptively transferred into wild-type BALB/c mice on day -1, followed by intraperitoneal or subcutaneous administration of mPEG-PLGA hydrogel containing OVA₃₂₃₋₃₃₉ (4 ng/mouse) with iloprost (5 µg/mouse) or vehicle on day 0. After 7 days, splenocytes or lymph node cells were stained with fluorochrome-labeled anti-CD3, CD4, CD25, and KJ126 clonotypic mAbs and analyzed using LSR II.

For *in vivo* Treg differentiation induced by iloprost-treated BM-DCs (Fig. 4), naïve CD4⁺ T cells (2×10^6 cells) from DO11.10 (Thy1.2⁺) mice were adoptively transferred into Thy1.1 BALB/c mice on day -1, followed by intravenous administration of OVA₃₂₃₋₃₃₉-pulsed BM-DCs (2×10^5 cells) on day 0. After 7 days, splenocytes from the recipient mice were stained with APC-anti-Thy1.2 (53–2.1; eBioscience), FITC-anti-KJ1-26 (KJ1-26; eBioscience), PE-Cy7-anti-CD3 (145-2c11; BD Biosciences), PerCP-anti-CD4 (RM4-5; eBioscience) and PE-anti-CD25 (PC61; BD Biosciences) and analyzed by flow cytometry (LSR II).

2.7. *In vitro* Treg functional assay

DO11.10 mice were intraperitoneally injected with 15% mPEG-PLGA hydrogel with OVA₃₂₃₋₃₃₉ (4 ng/mouse) and iloprost (5 ng/mouse) on day 0. On day 7, CD4⁺CD25⁺ Treg cells were purified from the spleen and cocultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained CD4⁺ effector T cells from DO11.10 mice at a ratio of 1:1 or 2:1 in the presence of anti-CD3/CD28 beads for 3 days. The percentage of dividing effector T cells was analyzed by flow cytometry (LSR II; BD Biosciences).

2.8. Statistical analysis

Statistical comparisons of data among control and experimental groups were performed with the nonparametric Mann-Whitney *U* test. Values of *p* < 0.05 were considered significant. All statistical tests were performed by GraphPad Prism 5.0.

3. Results

3.1. Iloprost suppressed airway allergic lung inflammation *in vivo*

To evaluate the anti-inflammatory effect of iloprost *in vivo*, we established an allergic lung inflammation mouse model by subcutaneously injecting a mixture of OVA/iloprost or OVA/vehicle with a thermosensitive biodegradable hydrogel, mPEG-PLGA hydrogel. mPEG-PLGA hydrogel is a widely used biomaterial system for drug delivery due to its slow and stable biodegradability and biocompatibility [29]. It has been demonstrated that this hydrogel system can codeliver Ag and cytokines to modulate DC function at the injection site [31]. In this model, we observed that OVA significantly induced Th2-mediated airway inflammation, including eosinophil recruitment, the secretion of IL-4, IL-5, and IL-13 in BALFs as well as immune cell infiltration and goblet cell proliferation in lung tissues. However, codelivering of OVA and iloprost resulted in fewer cardinal features of OVA-induced Th2 airway inflammation (Fig. 1). This finding suggests that PGI₂ signaling mediated by iloprost may modulate airway allergic inflammation *in vivo*.

3.2. Iloprost promoted Ag-specific adaptive Treg differentiation *in vivo*

A previous study reported that iloprost promoted *FOXP3* gene expression in cocultures of human monocyte-derived DCs and naïve CD4⁺ T cells *in vitro* [14], suggesting that iloprost may modulate allergic inflammation through the interaction between DCs and Foxp3⁺ Tregs.

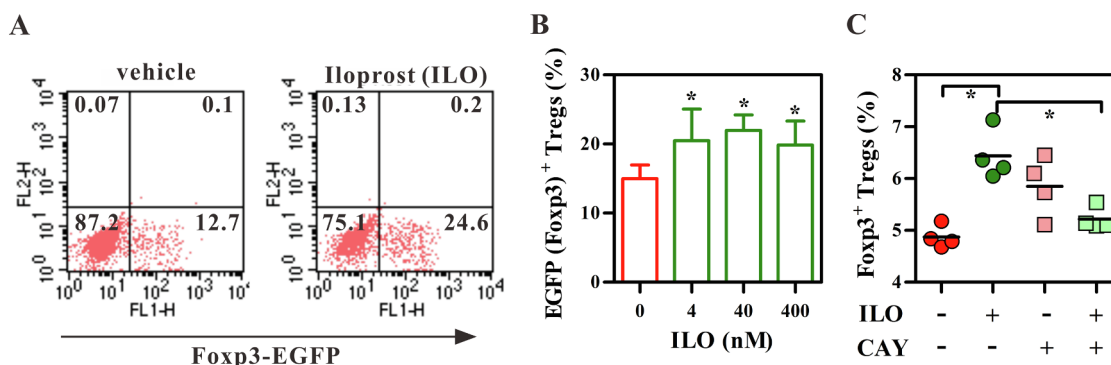


Fig. 3. Adaptive Treg differentiation *in vitro* induced by iloprost-treated BM-DCs. (A) Day-8 BM-DCs were treated with vehicle or iloprost (ILO) in the presence of LPS for 24 h. The harvested cells were cocultured with naïve CD4⁺ T cells from Foxp3^{EGFP} mice at a DC:T cell ratio of 1:10 in Treg-skewing conditions for 3 days. Representative dot-plots show the percentages of viable EGFP (Foxp3)⁺ CD4⁺ Tregs. The number in each figure represents the percentage of cell subsets. (B) Results are shown as percentages of EGFP (Foxp3)⁺ cells in gated CD3⁺CD4⁺ T cells. Data are the mean $\pm$ SD of five independent experiments. (C) Day-8 BM-DCs were treated with iloprost (40 nM) and LPS in the presence or absence of CAY10441 (1 µM) for 24 h. Treated BM-DCs were cocultured with naïve CD4⁺ T cells from wild type mice in Treg-skewing conditions for 3 days. Results are shown as percentages of Foxp3⁺ cells in gated CD3⁺CD4⁺ T cells. **p* < 0.05 compared with vehicle.

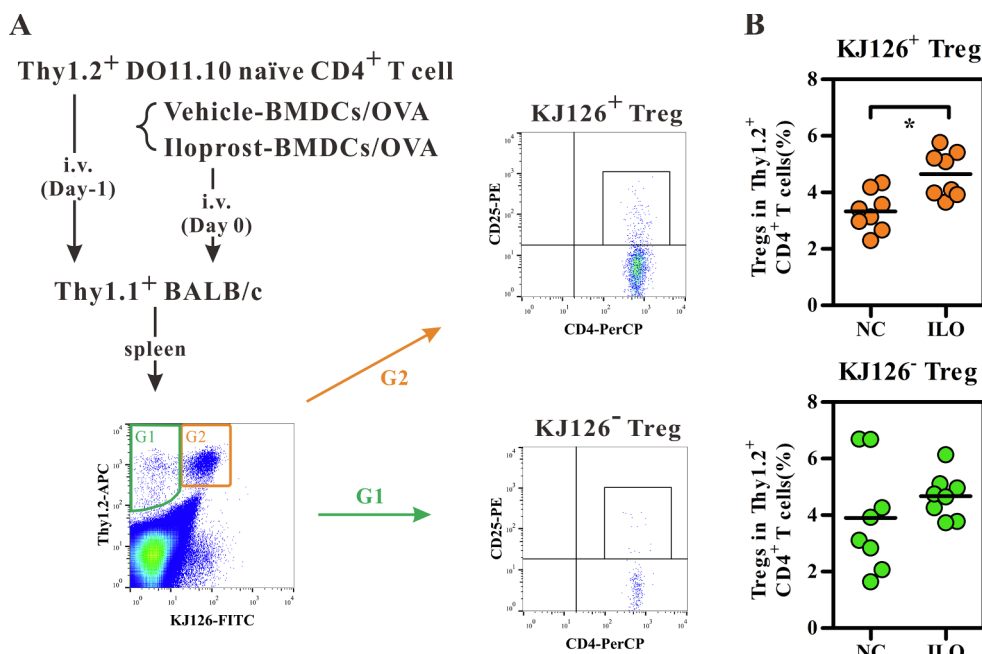


Fig. 4. Adaptive Treg differentiation *in vivo* induced by iloprost-treated BM-DCs. (A) Adoptive transfer experiments of donor naïve T cells (Thy1.2⁺ CD3⁺ CD4⁺ CD62L⁺) and treated OVA₃₂₃₋₃₃₉-pulsed BM-DCs into Thy1.1⁺ naïve mice for 7 days. Splenic donor Thy1.2⁺ Tregs were analyzed by flow cytometry. Both conditions of BM-DCs were treated with LPS. Representative dot plots show the expression of Thy1.2 on gated CD3⁺ CD4⁺ T cells (bottom) and the expression of donor Ag-specific Tregs (KJ126⁺ CD4⁺ CD25⁺; upper panel) or donor non-Ag-specific Tregs (KJ126⁻ CD4⁺ CD25⁺; lower panel). (B) The results are shown as percentages of donor Ag-specific Tregs or donor non-Ag-specific Tregs in splenocytes. Symbols represent the frequencies of Tregs in each individual mouse. The line within the vertical points marks the mean for each group. Data were from two independent experiments. NC: vehicle; ILO: iloprost. **p* < 0.05 compared with vehicle.

We next examined whether the iloprost effect was mediated by OVA-specific Treg differentiation *in vivo*. Adoptive transfer of DO11.10 naïve KJ126⁺ CD4⁺ T cells into syngeneic BALB/c mice was performed on day -1, followed by administration of iloprost/hydrogel either via the intraperitoneal or subcutaneous route on the following day (Fig. 2A). After 7 days, iloprost-treated mice showed a significantly higher frequency of KJ126⁺ CD4⁺ CD25⁺ Treg cells, but not of KJ126⁻ Tregs, either in the spleen through the intraperitoneal route or in local lymph nodes through the subcutaneous route (Fig. 2B and C). These results indicate that iloprost promotes Ag-specific adaptive Treg differentiation *in vivo*.

3.3. Iloprost-treated DCs displayed tolerogenic phenotypes *in vitro* and *in vivo*

Considering the key role of DCs in adaptive Treg differentiation, we speculated that iloprost may promote Treg differentiation by modulating DC function. First, we analyzed whether iloprost modulates DCs to display tolerogenic phenotypes. Consistent with a previous study [13], we observed that iloprost-treated BM-DCs secreted significantly less inflammatory cytokines, such as IL-6, IL-12, IL-23, and TNF- α , but more anti-inflammatory cytokines, such as IL-10, in a dose-dependent manner in response to LPS stimulation (Supplementary Fig. 1). In

addition, iloprost treatment inhibited the expression of CD40, CD80, CD86, and MHC class II in response to LPS stimulation (Supplementary Fig. 2). We further analyzed the maturity of iloprost-conditioned DCs *in vivo*. Using mPEG-PLGA hydrogel to slowly release iloprost *in vivo*, the less percentage of MHC class II⁺ cDCs (CD11c⁺ PDCA1⁻) in local lymph nodes has been observed in iloprost-treated mice during the first 6 days (Supplementary Fig. 3A). However, iloprost did not significantly affect the maturity of pDCs (CD11c⁺ PDCA1⁺) in local lymph nodes (Supplementary Fig. 3B). This suggests that iloprost-conditioned cDCs may have tolerogenic properties and can be considered TDCs either *in vitro* or *in vivo*.

3.4. Iloprost-treated DCs promoted adaptive Treg differentiation *in vitro* and *in vivo*

As iloprost-treated DCs have a tolerogenic phenotype, we next analyzed whether they can promote adaptive Treg differentiation through the IP receptor. *In vitro* coculture experiments showed that iloprost-treated BM-DCs significantly increased the frequency of EGFP⁺ Treg cells, which were differentiated from naïve Foxp3^{EGFP} CD4⁺ T cells in the presence of TGF- β (Fig. 3A and B). Treatment of BM-DCs with CAY10441, an antagonist for the IP receptor, abrogated the effect of iloprost on adaptive Treg differentiation (Fig. 3C). These results suggest

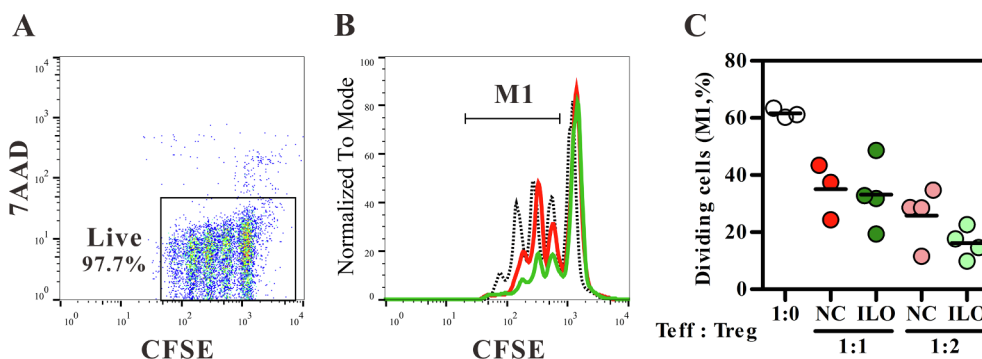


Fig. 5. The suppressive function of iloprost-induced Tregs *in vitro*. DO11.10 mice were subcutaneously injected with 15% mPEG-PLGA hydrogel combined with 5 ng iloprost and 4 ng OVA₃₂₃₋₃₃₉ peptide. After 7 days, Treg cells (CD4⁺ CD25⁺) were purified and cocultured with CFSE-labeled effector T cells at different ratios. (A) The proliferation status of effector T cells on day 3. (B) Effector T cells cocultured with Treg cells at a 1:2 ratio. Black: effector T cells only. Red: effector T cells cocultured with Tregs from control mice. Green: effector T cells cocultured with Tregs from iloprost-treated mice. (C) The percentages of dividing effector T cells.

effector T cells (M1 gate as shown in A) when they were cocultured with Tregs at different ratios. There is no significant difference between two groups at both ratios. One representative dataset was obtained from two independent experiments. NC: vehicle; ILO: iloprost. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that iloprost-treated DCs promoted adaptive Treg differentiation through the IP receptor. Furthermore, we tracked naïve CD4⁺ T cell differentiation by adoptively transferring Thy1.2⁺ DO11.10 naïve CD4⁺ T cells and iloprost-treated and OVA₃₂₃₋₃₃₉-pulsed BM-DCs into Thy1.1⁺ recipient mice (Fig. 4A). To avoid losing the difference of Ag-specific adaptive Treg frequencies between iloprost and control groups due to the complicated intracellular staining procedure for Foxp3, CD25 marker was used as a representative marker in this experiment as CD4⁺CD25⁺ T cells consist of more than 90% Foxp3⁺ cells in mice [32]. As shown in Fig. 4B, iloprost-treated BM-DCs significantly increased the percentage of donor Thy1.2⁺KJ126⁺ Tregs relative to the percentage of donor Thy1.2⁺KJ126⁻ Tregs in the spleen.

Next, we analyzed the suppressive function of iloprost-induced Tregs using CFSE-labeling experiments. As shown in Fig. 5, purified CD4⁺CD25⁺ Tregs induced by iloprost *in vivo* displayed similar suppressive ability in comparison with control Tregs at different effector T cell/Treg ratios. Taken together, these data suggest that iloprost-treated DCs not only have tolerogenic phenotypes but also have the ability to promote the adaptive differentiation of suppressive Tregs.

3.5. Iloprost-treated DCs promoted Treg differentiation, at least in part, through PD-L1

As iloprost-treated DCs have tolerogenic properties, we further explored the mechanism involved in this process. Flow cytometric analysis showed that iloprost-treated DCs significantly increased the expression of PD-L1 (Fig. 6A–C) and ICOS-L (Supplementary Fig. 4A and 4B), but not PD-L2 and SLAM (data not shown). In addition,

pretreatment of BM-DCs with CAY10441 abrogated the effect of iloprost on PD-L1 expression (Fig. 6D and E), suggesting that iloprost-induced PD-L1 expression in DCs was mediated by the IP receptor. Furthermore, the blocking experiment showed that blocking the PD-L1 interaction between iloprost-treated BM-DCs and naïve CD4⁺ T cells significantly suppressed the frequency of EGFP (Foxp3)⁺ Tregs, which were differentiated from naïve T cells (Fig. 6F). However, blocking ICOS-L or IL-10 in the coculture did not affect iloprost-treated BM-DC-induced adaptive Treg differentiation (Supplementary Fig. 4C and 4D). These data suggest that PD-L1 is, at least in part, responsible for the adaptive Treg differentiation mediated by iloprost-treated BM-DCs.

3.6. Iloprost treatment suppressed DC-mediated airway inflammation and enhanced Ag-specific Treg differentiation through PD-L1 *in vivo*

To further analyze the *in vivo* function of iloprost-treated DCs, BM-DCs without LPS and iloprost treatment were used as a positive control for adaptive Treg induction as it has been demonstrated that immature DCs can promote Treg differentiation [20]. As shown in Supplementary Fig. 5, intratracheal injection of immature BM-DCs significantly enhanced higher percentages of CD4⁺Foxp3⁺ Treg differentiation than the mature LPS-treated BM-DCs in local lymph nodes, whereas iloprost/LPS-treated DCs regained the tolerogenic property in Treg induction. Next, we asked whether iloprost treatment inhibited DC-mediated airway inflammation and promoted Ag-specific Treg differentiation through PD-L1. As shown in Fig. 7A and B, airway inflammation induced by LPS-treated BM-DCs was mediated both eosinophil- and neutrophil-infiltrated lung inflammation. In contrast, iloprost/LPS-

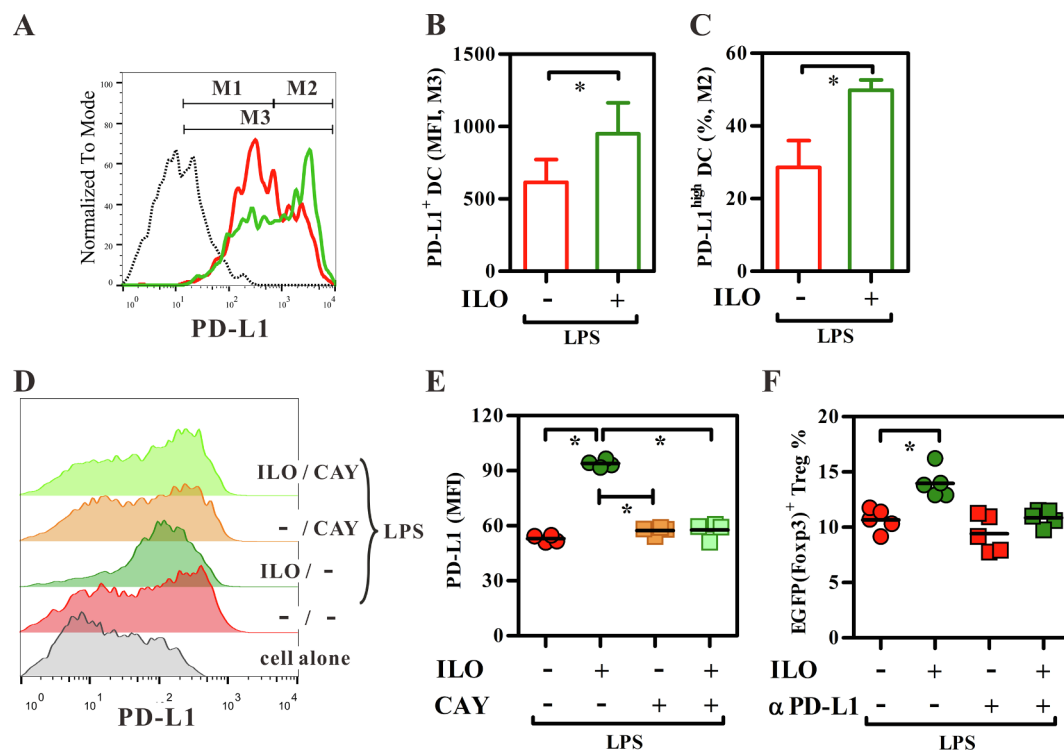


Fig. 6. PD-L1 expression on iloprost-treated BM-DCs. (A) Day-8 BM-DCs were treated with vehicle or iloprost (40 nM; ILO) in the presence of LPS for 24 h. The harvested cells were stained with fluorochrome-labeled antibodies for CD11c, PD-L1 and analyzed by flow cytometry. The histograms shown are gated on live CD11c⁺ cells (black, isotype control; red, LPS alone; green, iloprost + LPS). Representative data from three experiments are shown. The mean fluorescence intensity (MFI) of PD-L1⁺ DCs (M3 gate as shown in A) (B) or the percentages of PD-L1^{high} DCs (M2 gate as shown in A) (C) are shown as the mean $\pm$ SD of five independent experiments. (D) Representative histograms show PD-L1 expression pattern in the CD11c-gated BM-DCs in the presence or absence of CAY10441 (1 μ M) as indicated. BM-DC without any treatment (cell alone) is considered as negative control for LPS-induced maturation. (E) The MFI of PD-L1 are shown from four independent experiments. (F) The treated BM-DCs were cocultured with naïve CD4⁺ T cells from Foxp3^{EGFP} mice at a DC:T cell ratio of 1:10 for 3 days in the presence of TGF- β and blocking mAbs (isotype control or anti-PD-L1 IgG). The live, EGFP (Foxp3)⁺ Tregs were analyzed using flow cytometry. One dot represents one mouse. The line within the vertical points marks the mean for each group. **p* < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

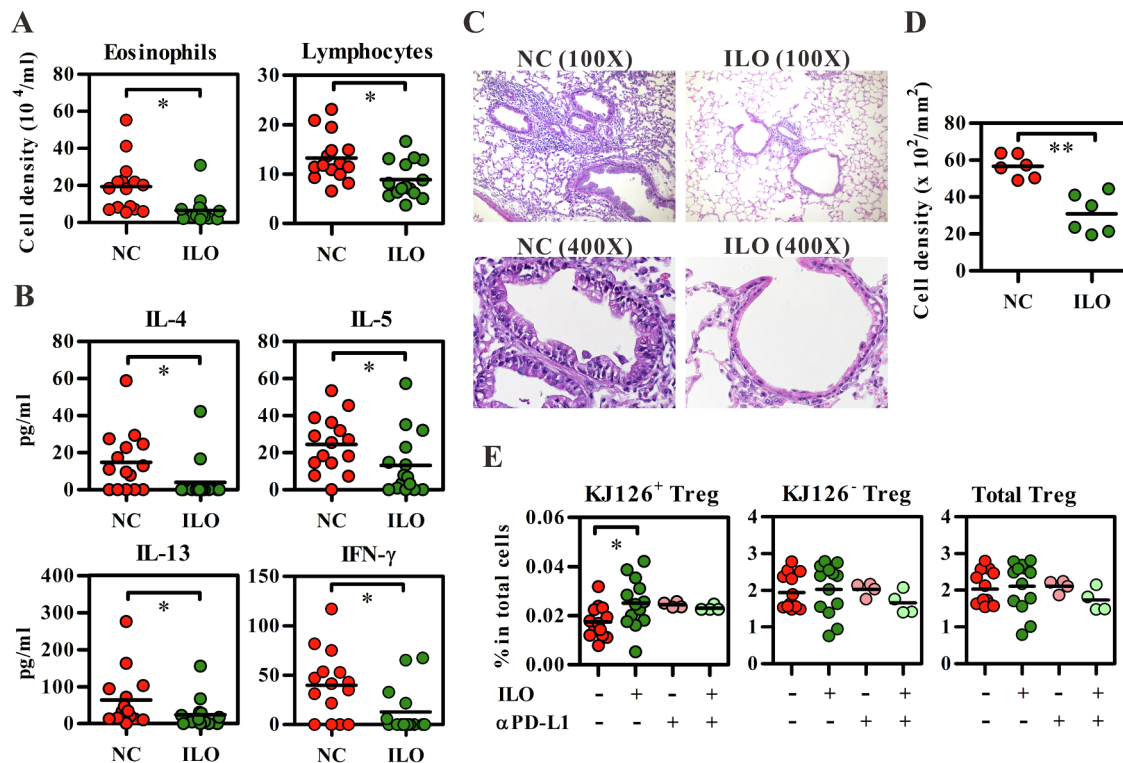


Fig. 7. Analysis of the inflammatory response and Treg frequency induced by iloprost-treated BM-DCs in a murine lung inflammation model. BALB/c mice were intratracheally injected with OVA-pulsed vehicle (NC) or iloprost-treated BM-DCs (ILO) on day 0 followed by daily OVA aerosol challenges on day 10–13. On the day following the last challenge, BALF cells (A), BALF cytokines (B), or lung tissue pathological changes (C) were analyzed by flow cytometry, ELISA or hematoxylin and eosin staining, respectively. (D) The density of hematoxylin⁺ cells in lung sections. (E) Analysis of Treg (CD3⁺CD4⁺Foxp3⁺) frequencies in BALF cells from BM-DC-treated mice as described above. The treated BM-DCs were incubated with anti-PD-L1 mAb prior to intratracheal injection into mice. All conditions of BM-DCs were treated with LPS. One dot represents one mouse. The line within the vertical points marks the mean for each group. NC: vehicle; ILO: iloprost. **p* < 0.05 or ***p* < 0.01 vs. vehicle.

treated BM-DCs induced inflammation to a lesser degree with low numbers of eosinophils and lymphocytes in BALFs as well as low IL-4, IL-5, IL-13 and IFN- γ expression levels when compared to LPS-treated BM-DCs. The pathological analysis also showed decreased infiltration of immune cells in lung tissues from the iloprost group (Fig. 7C and D). Most importantly, the frequency of KJ126⁺CD4⁺CD25⁺ (Ag-specific) Tregs in BALFs was significantly higher in the iloprost group than in the control group, whereas the iloprost-treated BM-DC-induced Ag-specific Treg differentiation was significantly inhibited by anti-PD-L1 blocking mAbs (Fig. 7E). However, the iloprost effect was not observed in the frequencies of non-Ag-specific Tregs and total Tregs (Fig. 7E). These results suggest that iloprost-treated DCs may control inflammation through Ag-specific adaptive Treg differentiation in a PD-L1-dependent manner.

4. Discussion

In the present study, we demonstrated PGI₂-IP signaling mediated by iloprost in DCs promotes Ag-specific Treg differentiation. Iloprost-treated DCs show tolerogenic features, including an increase in surface markers associated with tolerogenicity (PD-L1 and ICOS-L) and the anti-inflammatory cytokine IL-10 but a decrease in inflammatory cytokines (IL-6, IL-12, IL-23, TNF- α). Among these mediators/markers, PD-L1 seems to play an important role in iloprost-treated DC-mediated Ag-specific Treg differentiation. This study provides a potential drug candidate to develop a TDC therapy for autoimmune diseases or allergic asthma.

Although the PGI₂ receptor IP can be expressed both in DCs [33] and naive CD4⁺ T cells [12], PGI₂-IP signaling seems to induce opposing responses in different types of immune cells. In support of our

findings, Müller et al. showed that iloprost enhanced the anti-inflammatory properties of human myeloid DCs and promoted *FOXP3* gene expression in cocultures of DCs and CD4⁺CD45RA⁺ T cells *in vitro* [14]. Müller's study and our study demonstrate that PGI₂-IP signaling in DCs leads to an anti-inflammatory response; however, when PGI₂-IP signaling acts directly on naive CD4⁺ T cells in the presence of Th-skewing conditions, PGI₂-IP signaling enhances Th17 or Th1 differentiation to favor inflammatory responses [10,12]. On the other hand, PGI₂-IP signaling has also been shown to play an opposing role in various inflammatory disease models using IP-deficient mice. In an IP-deficient allergic asthma model, PGI₂-IP signaling plays a regulatory role by suppressing Th2-mediated lung inflammation [15,16]. However, PGI₂-IP signaling may enhance the onset of Th17-mediated neurological inflammation in an experimental autoimmune encephalomyelitis (EAE) model [10]. Additionally, PGI₂-IP signaling may initiate acquired cutaneous immune responses by promoting Th1 differentiation in a contact hypersensitivity model [33]. Taken together, these results suggest that the role of PGI₂-IP signaling in immunity may be determined by the context of the disease and the predominant type of target cells.

In the present study, we developed a novel allergic inflammation animal model using mPEG-PLGA hydrogel admixed with OVA and iloprost. mPEG-PLGA hydrogel is an FDA-approved thermosensitive biodegradable material that can be easily prepared in a liquid state at room temperature and form a solid at body temperature [29]. The advantages of applying mPEG-PLGA hydrogel to the living body include not only prolonging the time that the drug remains in the circulatory system but also enhancing the accumulation of the drug in the target tissue to effectively treat the disease [31,34,35]. Due to its physical features, we speculated that the polymeric micelle structure in mPEG-

PLGA hydrogel may slow the release of iloprost and OVA *in vivo*, leading to the simultaneous targeting of OVA and iloprost to local DCs at peripheral sites. Although mPEG-PLGA hydrogel did not appear to have an immune adjuvant effect compared to traditional aluminum hydroxide [36], the mixture of OVA and mPEG-PLGA hydrogel still effectively induced allergic inflammation through a DC-mediated Ag-specific immune response, as shown in the present study. The application of mPEG-PLGA hydrogel for drug delivery in TDC generation may provide new insights into inflammatory disease treatment.

To the best of our knowledge, PD-L1 can control inflammation through at least two mechanisms. In the first mechanism, PD-L1 plays a pivotal role in the development and function of induced Tregs [24,37,38]. In the second mechanism, PD-L1⁺ keratinocytes can suppress the effector T cell response *in vitro* and in a contact hypersensitivity murine model [39]. In the present study, we demonstrated that PGI₂-IP signaling-modulated DCs can promote adaptive Treg differentiation through PD-L1, but not through IL-10 or ICOS-L. However, the underlying mechanism for PGI₂-IP signaling-mediated PD-L1 expression in DCs requires further investigation. Taken together, our results suggest that PGI₂-IP-modulated DCs may have great therapeutic potential for inflammatory diseases.

PGI₂, or its analog, iloprost, is considered the most effective lipid mediator for treating PAH due to its ability to induce high levels of vasodilatation [40,41]. PGI₂ can activate cAMP/PKA through the GTP-binding protein G_s and alter the balance between Ca²⁺ and K⁺, leading to vascular smooth muscle cell hyperpolarization and relaxation [5,42]. In addition to the imbalance between vasodilation and vasoconstriction, PAH is also associated with altered homeostasis and function of Tregs and autoimmunity [43–45]. Tamosiuniene et al. demonstrated that the adoptive transfer of Tregs can effectively reduce disease severity in a PAH rat model [46], indicating the important role of Tregs in PAH. The evidence shown in the present study provides an alternative underlying therapeutic mechanism for iloprost or other PGI₂ analogs in PAH.

Funding

This work was supported by the fund NHRI-EX101-9824SC from National Health Research Institute, and by the funds MOST 105-2320-B-037-015, MOST 106-2314-B-037-072, MOST 107-2314-B-037-003, and MOST 108-2320-B-037-010 from Ministry of Science and Technology in Taiwan.

CRedit authorship contribution statement

Tzu-Hsuan Wong: Investigation, Formal analysis, Writing - original draft. **Rung-Jiun Gau:** Conceptualization, Investigation, Resources, Writing - original draft. **Yu-Fang Chen:** Investigation, Formal analysis. **Hsin-Hsin Shen:** Methodology, Resources. **Carl Tsai-Yu Lin:** Methodology, Resources. **Sen-Lu Chen:** Methodology, Resources. **Jau-Ling Suen:** Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

We thank the Center for Resources, Research and Development in KMU, Taiwan for providing LSRII and TissueFAXS.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2019.106106>.

References

- [1] V.K. Raker, M.P. Domogalla, K. Steinbrink, Tolerogenic dendritic cells for regulatory T cell induction in man, *Front. Immunol.* 6 (2015) 569, <https://doi.org/10.3389/fimmu.2015.00569>.
- [2] M.B. Lutz, Induction of CD4(+) regulatory and polarized effector/helper T cells by dendritic cells, *Immune Netw.* 16 (1) (2016) 13–25, <https://doi.org/10.4110/in.2016.16.1.13>.
- [3] G. Florez-Grau, I. Zubizarreta, R. Cabezon, P. Villoslada, D. Benitez-Ribas, Tolerogenic dendritic cells as a promising antigen-specific therapy in the treatment of multiple sclerosis and neuromyelitis optica from preclinical to clinical trials, *Front. Immunol.* 9 (2018) 1169, <https://doi.org/10.3389/fimmu.2018.01169>.
- [4] B.E. Phillips, Y. Garciafigueroa, M. Trucco, N. Giannoukakis, Clinical tolerogenic dendritic cells: exploring therapeutic impact on human autoimmune disease, *Front. Immunol.* 8 (2017) 1279, <https://doi.org/10.3389/fimmu.2017.01279>.
- [5] J.A. Mitchell, B. Ahmetaj-Shala, N.S. Kirkby, W.R. Wright, L.S. Mackenzie, D.M. Reed, et al., Role of prostacyclin in pulmonary hypertension, *Glob. Cardiol. Sci. Pract.* 2014 (4) (2014) 382–393, <https://doi.org/10.5339/gcsp.2014.53>.
- [6] M. Idzko, H. Hammad, M. van Nimwegen, M. Kool, N. Vos, H.C. Hoogsteden, et al., Inhaled iloprost suppresses the cardinal features of asthma via inhibition of airway dendritic cell function, *J. Clin. Invest.* 117 (2) (2007) 464–472, <https://doi.org/10.1172/JCI28949>.
- [7] R. Norregaard, T.H. Kwon, J. Frokiaer, Physiology and pathophysiology of cyclooxygenase-2 and prostaglandin E2 in the kidney, *Kidney Res. Clin. Pract.* 34 (4) (2015) 194–200, <https://doi.org/10.1016/j.krcp.2015.10.004>.
- [8] K. Hashimoto, B.S. Graham, M.W. Geraci, G.A. FitzGerald, K. Egan, W. Zhou, et al., Signaling through the prostaglandin I2 receptor IP protects against respiratory syncytial virus-induced illness, *J. Virol.* 78 (19) (2004) 10303–10309, <https://doi.org/10.1128/JVI.78.19.10303-10309.2004>.
- [9] W. Zhou, K. Goleniewska, J. Zhang, D.E. Dulek, S. Toki, M.T. Lotz, et al., Cyclooxygenase inhibition abrogates aeroallergen-induced immune tolerance by suppressing prostaglandin I2 receptor signaling, *J. Allergy Clin. Immunol.* 134 (3) (2014) 698–705e5, <https://doi.org/10.1016/j.jaci.2014.06.004>.
- [10] W. Zhou, J. Zhang, K. Goleniewska, D.E. Dulek, S. Toki, D.C. Newcomb, et al., Prostaglandin I2 suppresses proinflammatory chemokine expression, CD4 T cell activation, and STAT6-independent allergic lung inflammation, *J. Immunol.* 197 (5) (2016) 1577–1586, <https://doi.org/10.4049/jimmunol.1501063>.
- [11] T. Koya, H. Matsuda, S. Matsubara, N. Miyahara, A. Dakhama, K. Takeda, et al., Differential effects of dendritic cell transfer on airway hyperresponsiveness and inflammation, *Am. J. Respir. Cell Mol. Biol.* 41 (3) (2009) 271–280, <https://doi.org/10.1165/rcmb.2008-0256OC>.
- [12] S. Toki, K. Goleniewska, M.M. Huckabee, W. Zhou, D.C. Newcomb, G.A. Fitzgerald, et al., PGI(2) signaling inhibits antigen uptake and increases migration of immature dendritic cells, *J. Leukoc. Biol.* 94 (1) (2013) 77–88, <https://doi.org/10.1189/jlb.1112559>.
- [13] W. Zhou, K. Hashimoto, K. Goleniewska, J.F. O'Neal, S. Ji, T.S. Blackwell, et al., Prostaglandin I2 analogs inhibit proinflammatory cytokine production and T cell stimulatory function of dendritic cells, *J. Immunol.* 178 (2) (2007) 702–710, <https://doi.org/10.4049/jimmunol.178.2.702>.
- [14] A. Saei, J. Hadjati, Tolerogenic dendritic cells: key regulators of peripheral tolerance in health and disease, *Int. Arch. Allergy Immunol.* 161 (4) (2013) 293–303, <https://doi.org/10.1159/000350328>.
- [15] K. Nagao, H. Tanaka, M. Komai, T. Masuda, S. Narumiya, H. Nagai, Role of prostaglandin I2 in airway remodeling induced by repeated allergen challenge in mice, *Am. J. Respir. Cell Mol. Biol.* 29 (3 Pt 1) (2003) 314–320, <https://doi.org/10.1165/rcmb.2003-0035OC>.
- [16] Y. Takahashi, S. Tokuoka, T. Masuda, Y. Hirano, M. Nagao, H. Tanaka, et al., Augmentation of allergic inflammation in prostanoide IP receptor deficient mice, *Br. J. Pharmacol.* 137 (3) (2002) 315–322, <https://doi.org/10.1038/sj.bjp.0704872>.
- [17] C. Horton, K. Shanmugarajah, J. Fairchild, Harnessing the properties of dendritic cells in the pursuit of immunological tolerance, *Biomed J* 40 (2) (2017) 80–93, <https://doi.org/10.1016/j.bj.2017.01.002>.
- [18] D.H. Munn, M.D. Sharma, J.R. Lee, K.G. Jhaver, T.S. Johnson, D.B. Keskin, et al., Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase, *Science* 297 (5588) (2002) 1867–1870, <https://doi.org/10.1126/science.1073514>.
- [19] I.P. Lewkowich, N.S. Herman, K.W. Schleifer, M.P. Dance, B.L. Chen, K.M. Dienger, et al., CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function, *J. Exp. Med.* 202 (11) (2005) 1549–1561, <https://doi.org/10.1084/jem.20051506>.
- [20] K. Kretschmer, I. Apostolou, D. Hawiger, K. Khazaie, M.C. Nussenzweig, von Boehmer, Inducing and expanding regulatory T cell populations by foreign antigen, *Nat. Immunol.* 6 (12) (2005) 1219–1227, <https://doi.org/10.1038/ni1265>.
- [21] C. Brinster, M. Shevach, Bone marrow-derived dendritic cells reverse the anergic state of CD4+CD25+ T cells without reversing their suppressive function, *J. Immunol.* 175 (11) (2005) 7332–7340, <https://doi.org/10.4049/jimmunol.175.11.7332>.
- [22] R.A. Maldonado, H. von Andrian, How tolerogenic dendritic cells induce regulatory T cells, *Adv. Immunol.* 108 (2010) 111–165, <https://doi.org/10.1016/B978-0-12-380995-7.00004-5>.
- [23] M. Brenk, M. Scheler, S. Koch, J. Neumann, O. Takikawa, G. Hacker, et al., Tryptophan deprivation induces inhibitory receptors ILT3 and ILT4 on dendritic cells favoring the induction of human CD4+CD25+ Foxp3+ T regulatory cells, *J. Immunol.* 183 (1) (2009) 145–154, <https://doi.org/10.4049/jimmunol.0803277>.
- [24] R. Schmelzeisen, C. Frolich, Prevention of postoperative swelling and pain by

- dexamethasone after operative removal of impacted third molar teeth, *Eur. J. Clin. Pharmacol.* 44 (3) (1993) 275–277, <https://doi.org/10.1007/bf00271371>.
- [25] L. Menard, T. Cantaert, N. Chamberlain, S.G. Tangye, S. Riminton, J.A. Church, et al., Signaling lymphocytic activation molecule (SLAM)/SLAM-associated protein pathway regulates human B-cell tolerance, *J. Allergy Clin. Immunol.* 133 (4) (2014) 1149–1161, <https://doi.org/10.1016/j.jaci.2013.10.051>.
- [26] A. Ephrem, A.L. Epstein, G.L. Stephens, A.M. Thornton, D. GlassE, M. Shevach, Modulation of Treg cells/T effector function by GITR signaling is context-dependent, *Eur. J. Immunol.* 43 (9) (2013) 2421–2429, <https://doi.org/10.1002/eji.201343451>.
- [27] S.A. Redpath, N. van der Werf, A.M. Cervera, A.S. MacDonald, D. Gray, R.M. Maizels, et al., ICOS controls Foxp3(+) regulatory T-cell expansion, maintenance and IL-10 production during helminth infection, *Eur. J. Immunol.* 43 (3) (2013) 705–715, <https://doi.org/10.1002/eji.201242794>.
- [28] J.E. Pogoriler, S. Rich, S.L. ArcherA, N. Husain, Persistence of complex vascular lesions despite prolonged prostacyclin therapy of pulmonary arterial hypertension, *Histopathology* 61 (4) (2012) 597–609, <https://doi.org/10.1111/j.1365-2559.2012.04246.x>.
- [29] F.C. Luft, Iloprost for asthma, *J. Mol. Med. (Berl.)* 89 (1) (2011) 1–3, <https://doi.org/10.1007/s00109-010-0705-3>.
- [30] T.H. Wong, H.A. Chen, R.J. Gau, J.H. YenJ, L. Suen, Heme oxygenase-1-expressing dendritic cells promote Foxp3+ regulatory T cell differentiation and induce less severe airway inflammation in murine models, *e0168919*, *PLoS ONE* 11 (12) (2016), <https://doi.org/10.1371/journal.pone.0168919>.
- [31] H.Y. Chou, X.Z. Lin, W.Y. Pan, P.Y. Wu, C.M. Chang, T.Y. Lin, et al., Hydrogel-delivered GM-CSF overcomes nonresponsiveness to hepatitis B vaccine through the recruitment and activation of dendritic cells, *J. Immunol.* 185 (9) (2010) 5468–5475, <https://doi.org/10.4049/jimmunol.1001875>.
- [32] S. Sakaguchi, Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self, *Nat. Immunol.* 6 (4) (2005) 345–352, <https://doi.org/10.1038/ni1178>.
- [33] C.H. Kuo, C.H. Lin, S.N. Yang, M.Y. Huang, H.L. Chen, P.L. Kuo, et al., Effect of prostaglandin I2 analogs on cytokine expression in human myeloid dendritic cells via epigenetic regulation, *Mol. Med.* 18 (2012) 433–444, <https://doi.org/10.2119/molmed.2011.00193>.
- [34] Y. Zhang, Y. Huang, S. Li, Polymeric micelles: nanocarriers for cancer-targeted drug delivery, *AAPS PharmSciTech* 15 (4) (2014) 862–871, <https://doi.org/10.1208/s12249-014-0113-z>.
- [35] W. Liu, H. Li, X. Zhang, D. Wen, F. Yu, S. Yang, et al., Prostaglandin I2-IP signalling regulates human Th17 and Treg cell differentiation, *Prostaglandins Leukot. Essent. Fatty Acids* 89 (5) (2013) 335–344, <https://doi.org/10.1016/j.plefa.2013.08.006>.
- [36] H. Hogenesch, Mechanism of immunopotential and safety of aluminum adjuvants, *Front. Immunol.* 3 (2012) 406, <https://doi.org/10.3389/fimmu.2012.00406>.
- [37] L. Wang, K. Pino-Lagos, V.C. de Vries, I. Guleria, M.H. SayeghR, J. Noelle, Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells, *Proc. Natl. Acad. Sci. U. S. A.* 105 (27) (2008) 9331–9336, <https://doi.org/10.1073/pnas.0710441105>.
- [38] H.S. McGee, H. Yagita, Z. ShaoD, K. Agrawal, Programmed death-1 antibody blocks therapeutic effects of T-regulatory cells in cockroach antigen-induced allergic asthma, *Am. J. Respir. Cell Mol. Biol.* 43 (4) (2010) 432–442, <https://doi.org/10.1165/rcmb.2009-0258OC>.
- [39] P. Ritprajak, M. Hashiguchi, F. Tsushima, N. ChalermarpM, Azuma, Keratinocyte-associated B7-H1 directly regulates cutaneous effector CD8+ T cell responses, *J. Immunol.* 184 (9) (2010) 4918–4925, <https://doi.org/10.4049/jimmunol.0902478>.
- [40] H. Olschewski, G. Simonneau, N. Galie, T. Higenbottam, R. Naeije, L.J. Rubin, et al., Inhaled iloprost for severe pulmonary hypertension, *N. Engl. J. Med.* 347 (5) (2002) 322–329, <https://doi.org/10.1056/NEJMoa020204>.
- [41] P. Fitscha, B. Tiso, T. Kraiss, H. Sinzinger, Effect of iloprost on in vivo and in vitro platelet function in patients with peripheral vascular disease (PVD), *Adv. Prostaglandin Thromboxane Leukot. Res.* 17A (1987) 450–454.
- [42] B.H. MajedR, A. Khalil, Molecular mechanisms regulating the vascular prostacyclin pathways and their adaptation during pregnancy and in the newborn, *Pharmacol. Rev.* 64 (3) (2012) 540–582, <https://doi.org/10.1124/pr.111.004770>.
- [43] A. Huertas, C. Phan, J. Bordenave, L. Tu, R. Thuillet, M. Le Hires, et al., Regulatory T cell dysfunction in idiopathic, heritable and connective tissue-associated pulmonary arterial hypertension, *Chest* 149 (6) (2016) 1482–1493, <https://doi.org/10.1016/j.chest.2016.01.004>.
- [44] M.R. Nicolls, L. Taraseviciene-Stewart, P.R. Rai, D.B. BadeschN, F. Voelkel, Autoimmunity and pulmonary hypertension: a perspective, *Eur. Respir. J.* 26 (6) (2005) 1110–1118, <https://doi.org/10.1183/09031936.05.00045705>.
- [45] S. Gaowa, W. Zhou, L. Yu, X. Zhou, K. Liao, K. Yang, et al., Effect of Th17 and Treg axis disorder on outcomes of pulmonary arterial hypertension in connective tissue diseases, 247372, *Mediators Inflamm.* 2014 (2014), <https://doi.org/10.1155/2014/247372>.
- [46] R. Tamosiuniene, O. Manouvakhova, P. Mesange, T. Saito, J. Qian, M. Sanyal, et al., Dominant role for regulatory T cells in protecting females against pulmonary hypertension, *Circ. Res.* 122 (12) (2018) 1689–1702, <https://doi.org/10.1161/CIRCRESAHA.117.312058>.