

PGI₂ signaling inhibits antigen uptake and increases migration of immature dendritic cells

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ABSTRACT

PGI₂ signaling through IP inhibits allergen-induced inflammatory responses in mice. We reported previously that PGI₂ analogs decreased proinflammatory cytokine and chemokine production by mature BMDCs. However, whether PGI₂ modulates the function of immature DCs has not been investigated. We hypothesized that PGI₂ negatively regulates immature DC function and investigated the effect of PGI₂ analogs on immature BMDC antigen uptake and migration in vitro and in vivo. Immature BMDCs were obtained from WT and IPKO mice, both on a C57BL/6 background. The PGI₂ analog cicaprost decreased FITC-OVA uptake by immature BMDCs. In addition, cicaprost increased immature BMDC podosome dissolution, pro-MMP-9 production, cell surface CCR7 expression, and chemotactic migration toward CCL19 and CCL21, as well as chemokinesis, in an IP-specific fashion. These in vitro results suggested that cicaprost promotes migration of immature DCs from mucosal surface to draining LNs. This concept was supported by the finding that migration of immature GFP⁺ BMDCs to draining LNs was enhanced by pretreatment with cicaprost. Further, migration of immature lung DCs labeled with PKH26 was enhanced by intranasal cicaprost administration. Our results suggest PGI₂-IP signaling increases immature DC migration to the draining LNs and may represent a novel mechanism by which this eicosanoid inhibits immune responses. *J. Leukoc. Biol.* 94: 000–000; 2013.

Introduction

PGI₂ is a product of arachidonic acid metabolism [1, 2] and is currently used for treatment of pulmonary hypertension, [3, 4]. There is increasing evidence suggesting that PGI₂ may also be a therapeutic target in asthma. For instance, mice genetically deficient in the IP, a seven-transmembrane GPCR, have augmented allergic airway disease in both acute and chronic mouse models. IP-deficient mice with allergic airway inflammation have increased airways responsiveness, eosinophilia, goblet cell metapla-

sia, and CD4 Th2 cytokine expression compared to WT mice [5, 6]. These studies suggest that endogenous PGI₂ inhibits allergic inflammation and the cardinal features of asthma.

Allergic inflammation is a result of innate and adaptive-immune responses. The impact of PGI₂ on CD4 T cell adaptive-immune responses has been studied extensively, and PGI₂ analogs decreased Th2 cell recruitment to the lung and inhibited the expression of the Th2 cytokines IL-5 and IL-13 in vitro [7–9]. DCs are innate immune cells that mature as a result of antigen uptake and are essential to initiate naïve CD4 T cell responses. Two groups have investigated the ability of PGI₂ to regulate mature DC functions. In an in vivo study, Idzko and colleagues [10] reported that intratracheal administration of the PGI₂ analog iloprost at the time of airway antigen exposure inhibited the migration of mature DCs that had taken up antigen to regional LNs and reduced the capacity of the DC to generate antigen-specific Th2 immune responses. At the same time, our group [11] reported that PGI₂ analogs inhibited matured BMDCs' production of the proinflammatory cytokines IL-12p70 and TNF- α , and the ability of BMDCs to stimulate CD4 T cell proliferation and production of IL-5 and IL-13 in an antigen-dependent fashion.

These studies revealed that PGI₂ analogs suppressed the capability of mature DCs to generate allergic responses. However, the ability of PGI₂ to regulate the function of immature DCs has not been described and is the goal of this study. Geissman and colleagues [12] reported that DC migration and maturation can be independently regulated events and DC migration can occur without maturation. In addition, they found that immature DCs regulate immune responses. Whereas we [11] and others [10] have previously reported the effect of PGI₂ analogs on mature DC function, this is the first report of PGI₂ on the function of immature DCs. In this context, we investigated the ability of PGI₂ to regulate immature DC antigen uptake, costimulatory molecule expression, pro-MMP-9 expression, podosome dissolution, and the expression of chemokine receptors critical for migration of DCs to the T cell zone of regional LNs. Podosomes are focal adhesion structure that have a F-actin-rich core surrounded

Abbreviations: BM=bone marrow, BMDC=bone marrow-derived DC, DP1=PGD₂ receptor 1, E2/E4=PGE₂ receptor 2/4, IP=PGI₂ receptor, KO=knockout, LTB₄=leukotriene B₄, MMP=matrix metalloproteinase

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by a ring structure that contains vinculin and talin [13]. Podosomes are necessary for DC adherence to ECM in peripheral tissues to facilitate antigen uptake in those areas [14]. In the airways, podosomes provide the mechanism by which immature DCs reside intercalated between epithelial cells to sample antigens in the airway lumen. Once podosomes are dissolved, the DC is no longer tethered to the epithelium and can migrate to the regional LNs. In addition, MMPs have an essential role in DC egress from mucosal surfaces to draining LNs through degradation of ECM and basement membrane which allows DCs to move through the ECM-filled space in cell migration [15]. MMP-9 is especially important for DC accumulation in airway mucosal area, since it cleaves collagen IV, a major component of basement membranes [16]. DC migration to LNs is mediated by CCR7 expression on the surface of the DC that binds to the chemokines CCL19 and CCL21 that are produced by the stromal cells in the T cell zones of the LNs. This provides the mechanism by which DCs colocalize with naïve T cells that also express CCR7 on their surface [17, 18]. Our in vitro studies showed that PGI₂ analogs reduced immature DC antigen uptake, enhanced costimulatory molecule expression, enhanced podosome dissolution by immature DCs, induced pro-MMP-9 expression, up-regulated immature DC CCR7 surface expression, and increased the ability of immature DCs to migrate toward CCL19 and CCL21. In vivo, in two different models we found PGI₂ analog treatment increased immature DC migration to the draining regional LNs. These data show that PGI₂ inhibits DC-mediated immune activation by enhancing immature DC migration and also by decreasing antigen uptake. These results provide two potential mechanisms by which PGI₂ may be therapeutically beneficial in hypersensitivity diseases, such as asthma.

MATERIALS AND METHODS

Mice

IPKO mice were generated previously by homologous recombination in embryonic stem cells and were backcrossed to the C57BL/6 background over 10 generations [19]. Age- and sex-matched C57BL/6J mice, as WT control were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Female mice were used at 9–13 weeks of age. Mice with enhanced GFP under control of the chicken β -actin promoter and CMV enhancer, which leads to GFP expression in all cells, were obtained from Jackson Laboratory [20]. All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Reagents and antibodies

Cicaprost was a gift from Dr. Manuela Huebner (Schering, Berlin, Germany). Iloprost was obtained from Cayman Chemical (Ann Arbor, MI, USA). FITC-OVA was purchased from Life Technologies/Invitrogen (Carlsbad, CA, USA). Anti-CD11c Alexa Fluor647, anti-MHC class II (I-A/I-E) Alexa Fluor700, mouse CCL19-Fc fusion recombinant protein, and anti-human IgG-PE were purchased from eBioscience (San Diego, CA, USA). Anti-CCR5-PE, anti-CD40-FITC, anti-CD80-Pacific blue, and anti-CD86-PE-Cy7 were purchased from BioLegend (San Diego, CA, USA). Anti-vinculin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor488-labeled secondary antibody and Texas Red-conjugated phalloidin to stain F-actin were purchased from Life Technologies/Invitrogen. PKH26 Red fluorescent cell linker kit was purchased from Sigma-Aldrich.

Preparation of immature BMDCs

Immature BMDCs were generated using a method described previously with modifications [21]. BM cells were cultured in complete RPMI-1640 medium (Mediatech, Manassas, VA, USA), containing 5% heat-inactivated FBS (HyClone, Logan, UT, USA), 50 μ g/ml gentamicin, and 55 nM 2-ME. GM-CSF (R&D Systems, Minneapolis, MN, USA) was added to the cell suspension at 20 ng/ml. The cells were cultured in six-well plates (Costar, Corning, NY, USA) and maintained at 37°C in humidified air containing 5% CO₂. On Day 3, fresh complete medium containing 20 ng/ml GM-CSF was added. At Day 6, non- and loosely adherent cells were harvested. Greater than 80% of the harvested cells were CD11c⁺ cells. These cells were treated with PGI₂ analogs. Mature BMDCs were generated from the immature BMDCs by the stimulation with 1 μ g/ml LPS (*Escherichia coli* serotype 055:B5; Sigma-Aldrich).

Antigen uptake assay

On Day 6 of culture with GM-CSF, loosely adherent, immature BMDCs were harvested and resuspended at 1×10^6 cells/ml in complete RPMI-1640 medium without GM-CSF. Cells (1×10^6) in the medium were added to 24-well plates (BD Biosciences, San Jose, CA, USA) and treated with cicaprost or iloprost at 37°C in humidified air containing 5% CO₂. After 24 h, 10 μ g FITC-OVA was added and incubated for 1 h. After incubation, those cells were harvested, and FITC-OVA uptake was measured by flow cytometry.

Flow cytometry

After treatment with PGI₂ analogs and FITC-OVA, those cells were stained with anti-CD11c Alexa Fluor647, mouse CCL19-Fc fusion recombinant protein, and anti-human IgG-PE. The cells were analyzed using a LSR II flow cytometer (BD Biosciences). A total of 10,000 live CD11c-positive cell events as gated on PI-negative and CD11c-positive fractions were acquired. The percentage of cells that were FITC-OVA⁺ and expressed CD40, CD80, CD86, MHC II, CCR5, and CCR7 was analyzed.

Cell treatment by antagonist of cAMP-dependent protein kinases

Immature BMDCs were pretreated with vehicle or PKA inhibitor Rp-8-Br-cAMPS at 1 μ M or 100 μ M for 30 min. Thereafter, the cells were treated with 10 nM cicaprost for 24 h. CCR7 expression was evaluated by flow cytometry.

Fluorescence microscopy

On Day 6 of culture, loosely adherent immature BMDCs were harvested and 2.5×10^5 immature BMDCs were seeded on poly-L-lysine-coated coverslips and left to adhere for 3 h at 37°C in humidified air containing 5% CO₂. After stimulation with cicaprost or vehicle solution, the cells were washed with PBS twice, fixed with 4% PFA in PBS for 10 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 2% BSA in PBS for 30 min. The cells were incubated at room temperature with anti-vinculin antibody for 1 h. Subsequently, the cells were washed with PBS and then incubated with Alexa Fluor488-labeled secondary antibody for 45 min. Finally, cells were washed in PBS and then incubated with Texas Red-conjugated phalloidin for 30 min. Cell images were collected on a confocal microscope (LSM 510 META) using a Plan-Apochromat 63 $\times$ /1.4 oil-immersion lens and LSM 510 software (Carl Zeiss, Jena, Germany), as described previously [22]. The number of cells expressing podosomes was counted in six images for each experimental condition; 150–250 cells were counted for each condition.

Chemotaxis and chemokinesis assay

Chemotaxis of immature BMDCs in response to CCL19 and CCL21 was measured in 24-well plates carrying transwell-permeable supports with a 5 μ m pore-size polycarbonate membrane (Costar). On Day 6 of culture, loosely adherent immature BMDCs were harvested and stimulated with cicaprost for 24 h. The upper chamber was loaded with 2.5×10^5 cells in 300 μ l RPMI 1640 containing 5% FBS. The lower chamber was filled with 700 μ l RPMI 1640 con-

taining 5% FBS with or without 100 ng/ml rCCL19 or rCCL21 (PeproTech, Rocky Hill, NJ, USA). After 2 h of incubation at 37°C, the migrating cells in the lower chamber were counted. A checkerboard assay to evaluate chemotaxis (directional migration) and chemokinesis (random migration) was performed by placing 100 ng/ml rCCL21 in the lower chamber only (chemotaxis and chemokinesis), or in the upper chamber only (chemokinesis), or both (chemokinesis) as described previously [23]. After incubation of the chambers for 2 h at 37°C, the migrating cells were collected from the lower chamber and counted.

Cytokine and MMP-9 measurement

The amounts of IL-12p70, IL-10, and pro-MMP-9 in the culture supernatants of BMDCs treated with cicaprost or LPS (1 µg/ml) for 24 h were measured by using mouse Quantikine ELISA kits (R&D Systems) following the manufacturer's instructions.

In vivo GFP-expressing BMDC migration assay

GFP expressing immature BMDCs were generated from GFP-mouse BM using the same protocol as described above. On Day 6 of culture with GM-CSF, the immature BMDCs were stimulated with 10 nM cicaprost or distilled water as a vehicle control for 24 h. Cells (2.5×10^6) in 30 µl PBS were then injected s.c. in the hind-leg footpad of a WT mouse. Cicaprost-treated GFP expressed immature BMDCs were administered into the right-hind footpad, while distilled water-treated cells were administered to the contralateral footpad. Some 6 and 12 h later, popliteal LNs were harvested and digested with collagenase (1 mg/ml; Sigma-Aldrich) and DNase I (0.2 mg/ml; Sigma-Aldrich) in RPMI 1640 with 5% FBS for 30 min and a single-cell suspension was generated by grinding and passing the material through a cell strainer. The cells were stained with CD11c antibody and PI. The number of migrating GFP expressed immature BMDCs was counted by flow cytometry.

In vivo lung DC migration assay by cell-linker administration

WT mice were anesthetized with ketamine and xylazine i.m., and then 80 µl of 20 µM PKH26 (Sigma-Aldrich) was instilled, 24 h before cicaprost (2.5 µg/60 µl) or PBS (60 µl) intranasal instillation. Mediastinal LNs were harvested 12 h later and digested with collagenase (1 mg/ml; Sigma-Aldrich) and DNase I (0.2 mg/ml; Sigma-Aldrich) in RPMI 1640 with 5% FBS for 30 min and a single-cell suspension was generated by grinding and passing the material through a cell strainer. The cells were stained with anti-CD11c antibody and PI. The number of PKH26-labeled immature BMDCs in the mediastinal LNs was counted by flow cytometry.

Statistical analysis

The *P* values were calculated by using the Mann-Whitney U-test. Values of *P* < 0.05 were considered significant.

RESULTS

PGI₂ analogs decreased FITC-OVA uptake by immature BMDCs in an IP-specific fashion

We first determined the surface phenotype of the immature BMDCs by flow cytometric analysis. Cells were cultured with GM-CSF for 6 days and then treated with or without 1 µg/ml LPS for 24 h. BMDCs treated with LPS specifically expressed high levels of surface MHC class II and the costimulatory molecules CD40, CD80, and CD86, characteristics of mature DCs. In contrast, those cells cultured with no LPS exhibited very low levels of MHC class II, CD40, CD80, and CD86, characteristics of immature DCs (Fig. 1). Data shown in Fig. 1 are representative of three independent experiments. We used the immature BMDCs

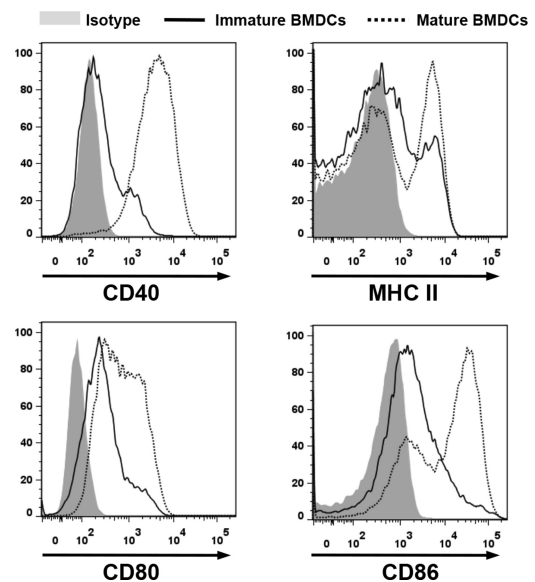
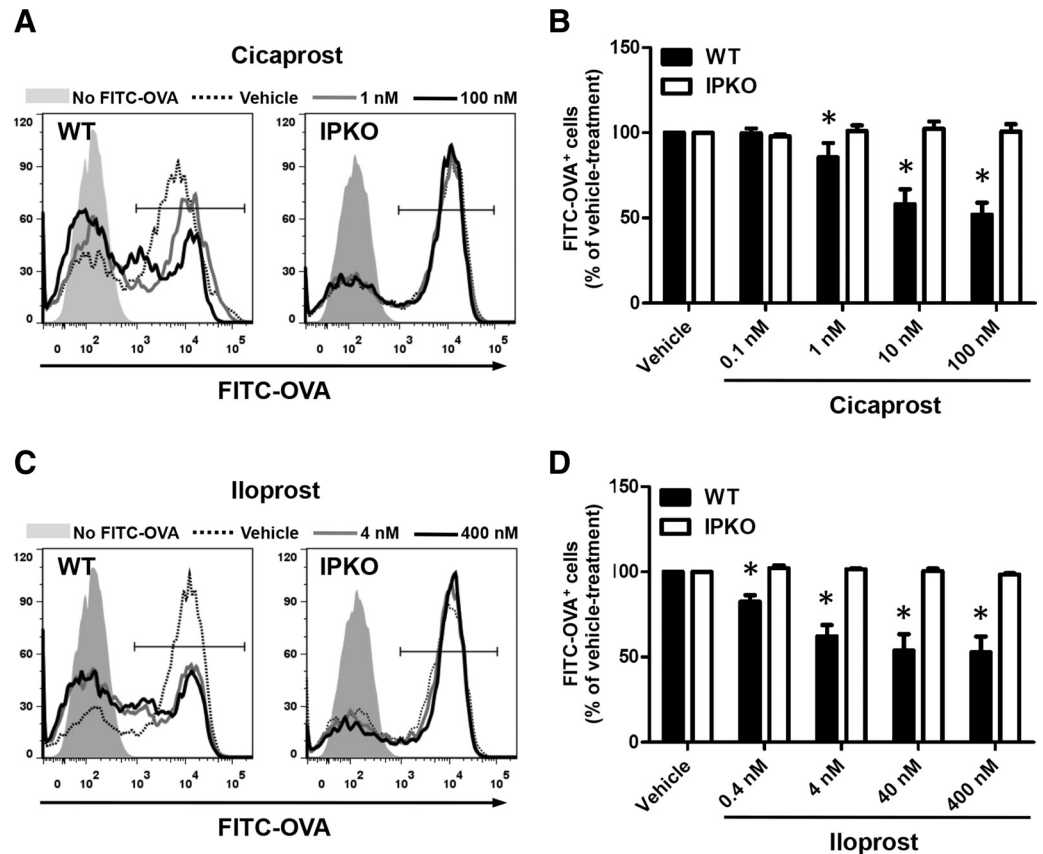


Figure 1. Phenotype of immature and mature BMDCs. BMDCs from WT mice were cultured in the absence (immature DC; bold lines) or presence (mature DC; dotted lines) of 1 µg/ml LPS for 24 h, and then cells were collected, washed, and analyzed by flow cytometry for surface expression of CD40, CD80, CD86, and MHC II. Filled gray histograms represent staining with isotype-matched control antibodies. One representative experiment of three is shown.

that were not treated with LPS in the remainder of the experiments in this study.

We have reported previously that PGI₂ analogs decreased the ability of mature DCs to activate T cells [11]. However, the effect of PGI₂ on antigen uptake by immature DCs has not been reported. We hypothesized that PGI₂ decreased antigen uptake by immature DCs. To test this hypothesis, immature BMDCs cultured with GM-CSF for 6 days were treated with either PGI₂ analogs or vehicle for 24 h. Next, FITC-OVA antigen was added to those cells for 1 h, and the intensity of FITC in immature BMDCs was assessed by flow cytometry. Viable DCs were defined as PI⁻ and CD11c⁺ cells. Treatment with the PGI₂ analog cicaprost significantly decreased the FITC⁺ peak in CD11c⁺ cells compared to vehicle-treatment (Fig. 2A) and the effect was dependent on the concentration of cicaprost. In contrast, cicaprost had no effect on FITC-OVA uptake by immature BMDCs derived from IPKO mice (Fig. 2A), revealing that the effect of cicaprost on antigen uptake was dependent on signaling through IP. FITC-positive cells were also decreased significantly on cicaprost-treated WT BMDCs compared to vehicle-treated DCs in a dose-dependent manner (Fig. 2B). Another PGI₂ analog, iloprost, also significantly decreased the FITC⁺ peak in CD11c⁺ cells compared to vehicle-treatment (Fig. 2C). Similar to cicaprost, iloprost also had no effect on antigen uptake by immature IPKO BMDCs at any of the concentrations examined (Fig. 2D). These results suggest that PGI₂ analogs inhibited antigen uptake by immature BMDCs in an IP-specific manner. As cicaprost and iloprost showed the same effect on immature BMDCs, the effects of cicaprost were described in following studies.

Figure 2. PGI₂ analogs decrease antigen uptake by immature BMDCs from WT mice, but not IPKO mice. Immature BMDCs from WT mice and IPKO mice were treated with vehicle, different concentrations of cicaprost and iloprost for 24 h. The cells were then treated with 10 μ g FITC-OVA for 1 h. The uptaken FITC-OVA was detected by flow cytometry in BMDCs from WT and IPKO mice treated with vehicle and either cicaprost (A) or iloprost (C). Bar graphs show the effect of cicaprost (B) and iloprost (D) as a percentage of vehicle-treatment. Values represent the mean $\pm$ SEM of three independent experiments. * P < 0.05 compared with the value of vehicle-treated cells.



Cicaprost induced costimulatory molecule expression but decreased CCR5 expression on immature BMDCs in an IP-specific fashion

DCs are professional APCs that have the capability to induce antigen-specific adaptive immunity, and mature DCs have high levels of surface expression of MHC and costimulatory molecules that are essential for effector T cell stimulation. We have shown that PGI₂ analogs decreased the ability of antigen uptake by immature BMDCs (Fig. 2), thus we investigated whether a PGI₂ analog alters the cell-surface expression of costimulatory molecules and MHC class II on immature BMDCs. Treatment of immature WT BMDCs with cicaprost for 24 h resulted in a statistically significant up-regulation of CD40, CD80, CD86, and MHC class II compared to vehicle-treatment. This is in contrast to our previously reported studies where cicaprost decreased cell surface expression of CD40, CD86, and MHC II on LPS-activated mature BMDCs. Cicaprost had no effect on the cell-surface expression of these molecules in immature IPKO BMDCs (Fig. 3A and B). These results reveal that cicaprost-treatment had the opposite effect on costimulatory molecule expression on immature and mature BMDCs. Cicaprost significantly decreased CCR5 expression on BMDCs from WT mice compared to vehicle-treatment while CCR5 expression on BMDCs from IPKO mice was not affected by cicaprost (Fig. 3A and B). Further immature BMDCs treated with cicaprost did not produce IL-12p70 and IL-10, whereas LPS-treated BMDCs produced significantly greater levels of these cytokines (Fig. 3C).

Cicaprost induced podosome dissolution by immature BMDCs in an IP-specific fashion

As a result of antigen uptake, DCs mature and migrate to regional LNs to present antigen to T cells. Idzko and colleagues [10] reported previously that iloprost administration inhibited migration of lung DCs that had taken up FITC-OVA, therefore we hypothesized that PGI₂ analog treatment would also down-regulate immature DC migration by negatively regulating podosome dissolution. We first examined podosome expression in immature DCs. As shown in Fig. 4, the majority of immature BMDCs displayed podosomes. These are visualized as F-actin (red)- and vinculin (green)-rich rings on the surface of immature BMDCs. Next, we determined whether cicaprost regulated podosome dissolution in immature DCs. The number of cells that expressed podosomes was counted in six images for each experimental condition and corresponds to 150–250 cells for each condition. Cicaprost-treatment for 1 h significantly induced podosome dissolution in immature WT BMDCs compared to vehicle-treatment in a dose-dependent manner (Fig. 5A and C). In addition, most podosomes in immature WT BMDCs were promptly dissolved within 15 min by 100 nM cicaprost (Fig. 5B and D). However, podosome dissolution in immature IPKO BMDCs was not observed with any concentration of cicaprost. Our results show that PGI₂-IP signaling induces podosome dissolution in immature DCs, and may facilitate the migration of immature DCs away from mucosal surfaces, where they ordinarily reside to sample environmental antigens.

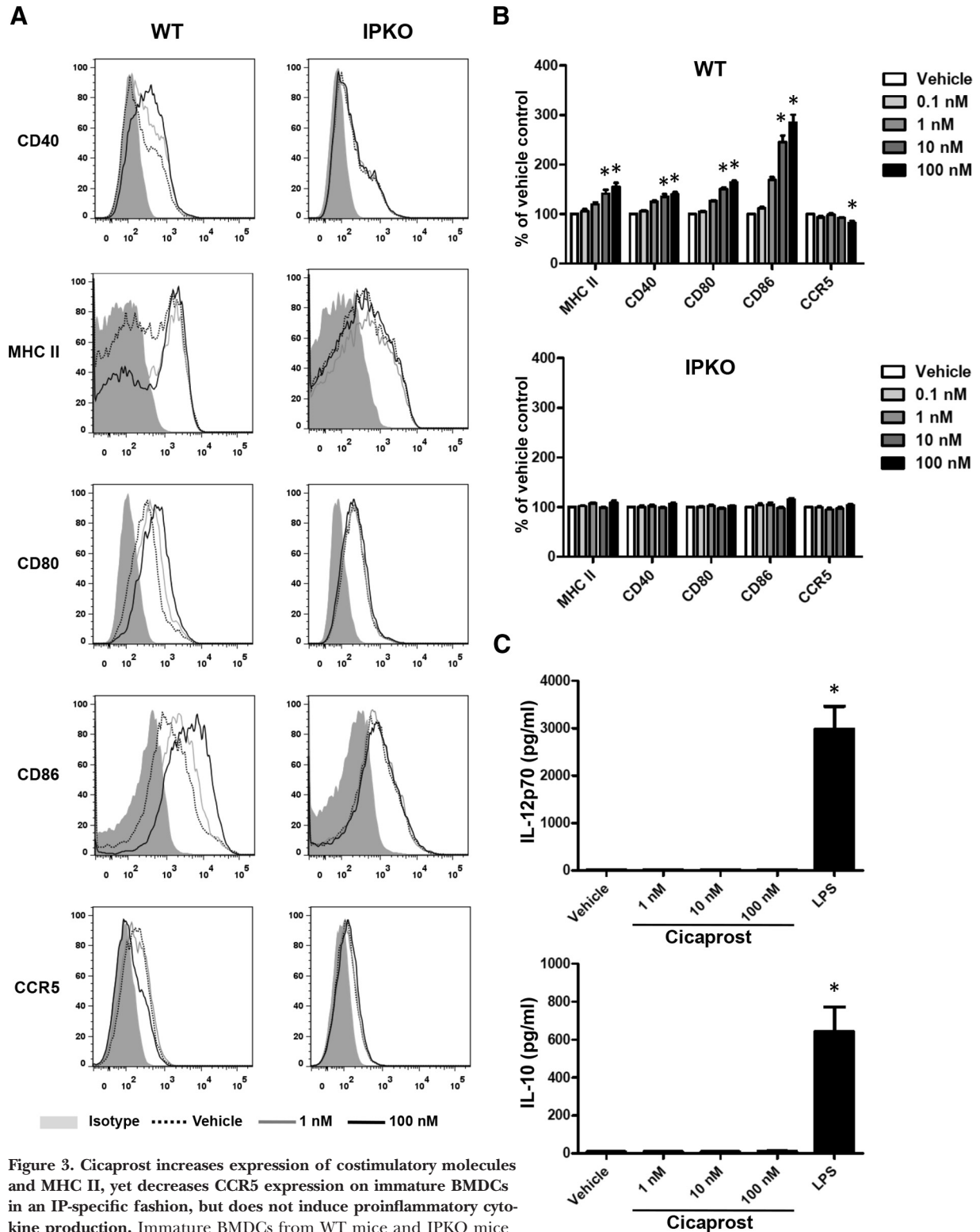


Figure 3. Cicaprost increases expression of costimulatory molecules and MHC II, yet decreases CCR5 expression on immature BMDCs in an IP-specific fashion, but does not induce proinflammatory cytokine production. Immature BMDCs from WT mice and IPKO mice were treated with vehicle and different concentrations of cicaprost for 24 h, and then cells were collected, washed, and analyzed by flow cytometry for the surface expression of CD40, CD80, CD86, MHC II, and CCR5. (A) Filled, gray histograms represent staining with isotype-matched control antibodies. One representative experiment of three is shown. (B) Bar graphs show the percentage of vehicle-treatment. Values represent the mean $\pm$ SEM of four independent experiments. (C) BMDCs from WT mice were treated with vehicle, different concentrations of cicaprost or 1 μ g/ml LPS for 24 h. Cytokine levels in culture supernatants were measured by ELISA. Values represent the mean $\pm$ SEM of four independent experiments. * P < 0.05 compared with the value of vehicle.

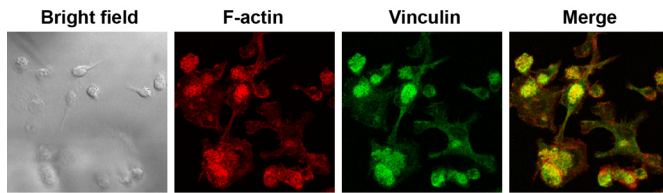


Figure 4. Immature BMDCs form podosomes as F-actin and vinculin rich section. BMDCs were cultured on poly-L-lysine-coated coverslips for 3 h and stained with an anti-vinculin antibody (green) and phalloidin-Texas Red (red) to detect F-actin.

Cicaprost increased CCR7 expression on immature BMDCs in an IP-specific fashion

As cicaprost enhanced podosome dissolution, we determined the ability of this PGI₂ analog to regulate expression of CCR7 on immature DCs and thus, potentially influence migration of these cells to the draining regional LNs. Cicaprost-treatment increased CCR7 expression significantly on CD11c⁺ immature WT BMDCs compared to vehicle-treatment (Fig. 6A and B). In contrast, cicaprost had no effect on CCR7 expression by immature IPKO BMDCs (Fig. 6A and B).

As PGI₂-IP signaling has been shown to increase intracellular cAMP, we investigated whether PGI₂-IP signaling regulates immature BMDC CCR7 expression in a cAMP-dependent manner. Pre-treatment of immature WT BMDCs with the cAMP antagonist Rp-8-Br-cAMPS suppressed cicaprost-induced CCR7 expression (Fig. 6C). These results indicate that PGI₂-IP signaling up-regulates CCR7 expression by immature DCs and thus may increase migration of immature DCs from sites of antigen sampling toward draining regional LNs.

Cicaprost induced immature BMDC chemotactic migration toward CCL19 and CCL21, as well as chemokinesis in vitro

As just shown, we found that PGI₂-IP signaling increased the CCR7 expression on immature BMDCs. To determine whether this PGI₂-driven increase in CCR7 expression is functional in altering the ability of immature DCs to respond to CCR7 ligands CCL19 and CCL21 that are expressed in the T cells zones of LNs, we quantified the migration of cicaprost- and vehicle-treated immature BMDCs toward these chemokines in a transwell migration assay. Cicaprost-treatment significantly increased the number of immature BMDCs that migrated to the chamber containing either CCL19 or CCL21 compared to vehicle-treatment in a dose-dependent manner (Fig. 7A). In contrast, the number of immature IPKO BMDCs that migrated to either CCL19 or CCL21 was not increased at any of the cicaprost concentrations examined (Fig. 7B). Checkerboard experiments were performed to clarify whether cicaprost-treatment influences chemotactic migration toward CCR7 ligand or chemokinetic migration. On checkerboard analyses, the presence of CCL21, only in the lower chamber represents chemotaxis and chemokinesis. On the other hand, the absence of CCL21 in the both upper and lower chambers, the presence of CCL21 in the upper chamber only, or the presence of CCL21 in both upper and lower chambers represent chemoki-

nosis as random migration. Cicaprost-treatment (10 nM) significantly increased the number of migrated immature WT BMDCs when CCL21 was in the lower chamber only and this was significantly greater than when CCL21 was in the upper and lower chambers (Fig. 7C). In contrast, the number of migrated vehicle-treated immature BMDCs was the same when CCL21 was only in the lower chamber and when CCL21 was in the upper and lower chambers. These results suggest that 10 nM cicaprost-treatment increased chemotaxis of immature WT BMDCs toward CCL21. However, 10 nM cicaprost-treatment also increased migration of immature WT BMDCs compared to vehicle-treated, immature WT BMDCs when CCL21 was in the upper and lower chambers, suggesting that cicaprost also increases chemokinesis (Fig. 7C). In contrast, the number of migrated immature IPKO BMDCs was not changed by cicaprost-treatment (Fig. 7D). Taken together, cicaprost significantly increased CCR7 expression on the surface of immature BMDCs and significantly increased the migration to biologically relevant ligands, while also increasing chemokinesis. This result supports the concept that PGI₂ may enhance migration of immature DCs away from environmental mucosal surfaces toward draining LNs.

Cicaprost induced MMP-9 production by immature BMDCs in an IP-specific fashion

MMP-9 is an important mediator of DC migration. To determine the effects of cicaprost on the expression of MMP-9, we measured pro-MMP-9 from the supernatant of immature BMDCs treated with cicaprost for 24 h. Cicaprost-treatment significantly increased pro-MMP-9 production by immature WT BMDCs compared to vehicle-treatment (Fig. 8). In contrast, cicaprost had no effect on pro-MMP-9 production by immature IPKO BMDCs (Fig. 8). This result supports that PGI₂ enhances immature DC migration.

Cicaprost induced immature BMDC migration to LNs in vivo

Cicaprost increased the cell surface expression of CCR7 on immature BMDCs and this was associated with increased migration of the cicaprost-treated, immature BMDCs to chemokines produced in LNs. Therefore, we hypothesized that immature BMDCs treated with a PGI₂ analog have enhanced migration to regional LNs compared to vehicle-treated immature BMDCs. To test this hypothesis, we isolated GFP expressing immature BMDCs and treated these cells with either cicaprost or vehicle for 24 h before washing the cells three times to remove any remaining cicaprost. We then injected these cells into the footpad of WT mice. Six hours and 12 h after the footpad injection, we harvested the popliteal LNs and performed flow cytometry on the cells to quantify the number of GFP expressing immature BMDCs that had migrated to the popliteal LNs (Fig. 9A). At 6 h, there was a twofold increase in the number of GFP expressing immature BMDCs that had been pretreated with cicaprost in the popliteal LNs compared to the cells that had been pretreated with vehicle; however, this difference was not statistically significant. However, 12 h af-

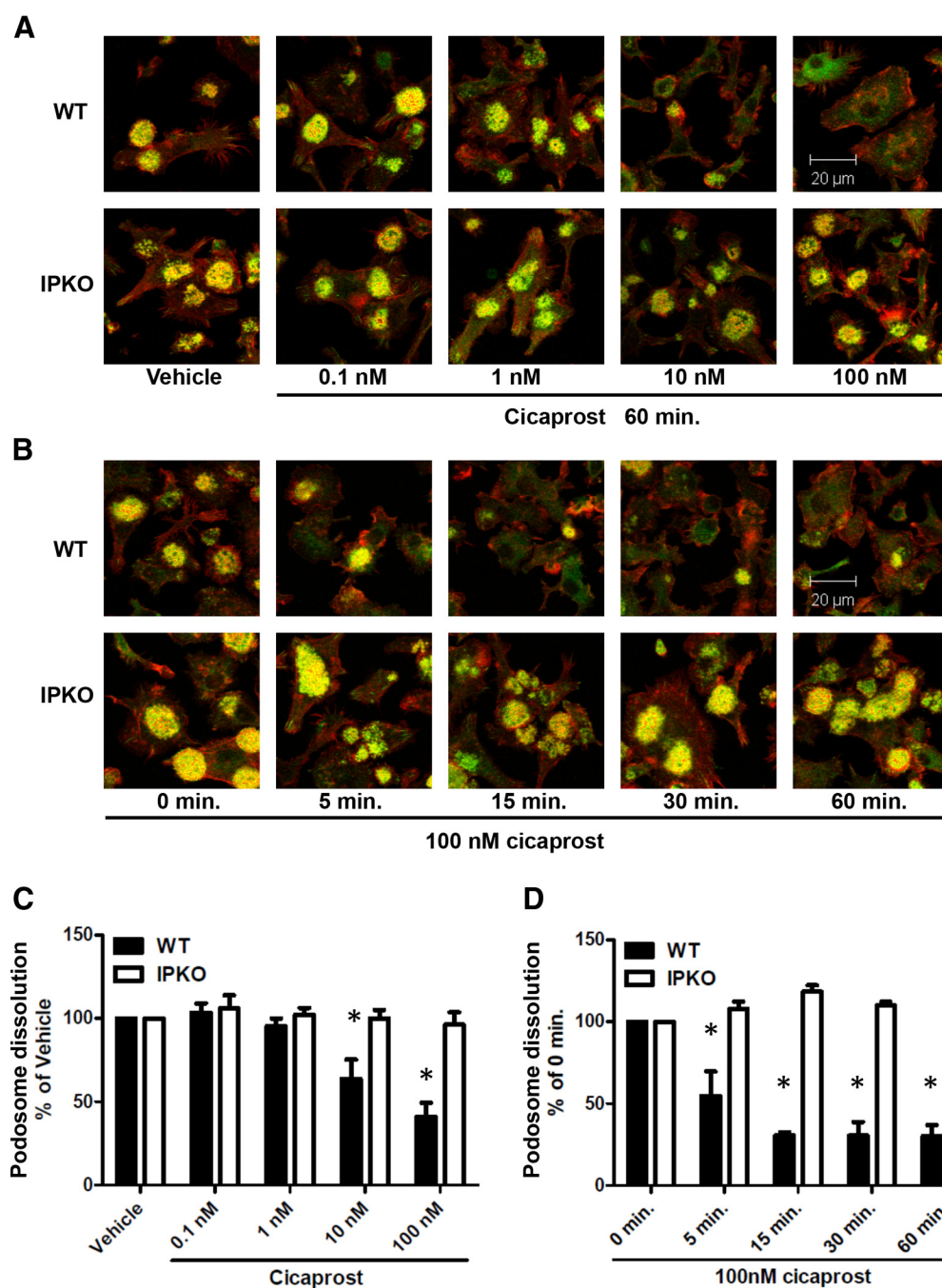


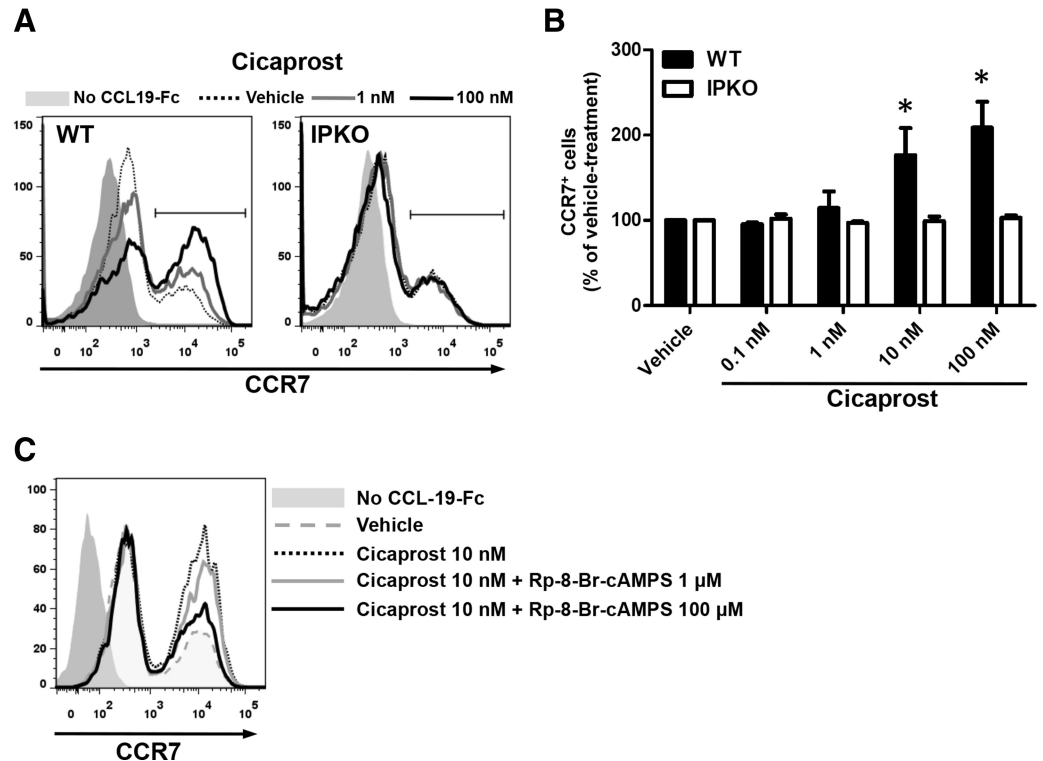
Figure 5. Cicaprost promotes podosome dissolution in immature BMDCs in an IP-specific fashion. (A) Immature BMDCs on coverslip were treated with vehicle and cicaprost at 0.1, 1, 10, and 100 nM for 60 min. The cells were then stained with an anti-vinculin antibody (green) and phalloidin-Texas Red (red). (B) Immature BMDCs on coverslip were untreated (0 min) or treated with 100 nM cicaprost for 5, 15, 30, or 60 min. (C) Bar graph shows effect of increasing concentrations of cicaprost on podosome dissolution. (D) Bar graph shows time course of podosome dissolution after treatment with 100 nM cicaprost. Values represent the mean $\pm$ SEM of three independent experiments. * $P < 0.05$ compared with the value of vehicle-treated samples or 0 min samples.

ter footpad injection, there was a statistically significant 2.5-fold increase in the number of GFP-expressing immature BMDCs that had been pretreated with cicaprost in the popliteal LNs compared to the cells that had been pretreated with vehicle (Fig. 9B). When the same experiment was performed and popliteal LNs were harvested at 24 and 48 h after injection of GFP expressing immature DCs, no GFP expressing cells were present in the popliteal LNs (data not shown). These results show that PGI₂-IP signaling increased immature DC migration from peripheral tissues to LNs.

We also assessed the ability of cicaprost to modulate immature DC migration in a different experimental model in a dif-

ferent organ system. In this experiment we administrated PKH26 cell linker intranasally to label DCs in the lung 24 h prior to intranasal cicaprost administration. PKH26 does not induce airway inflammation after administration [24]; therefore, we hypothesized that cicaprost administration increases the migration of immature DCs from the lung to the mediastinal LNs. As shown in Fig. 10A and B, cicaprost administration significantly increased the number of PKH26⁺ CD11c⁺ DCs in mediastinal LNs compared to PBS administration. This result supports the concept that PGI₂-IP signaling increased lung resident immature DC migration toward mediastinal LNs in steady state.

Figure 6. Cicaprost increases CCR7 expression on immature BMDCs from WT mice, but not IPKO mice through a cAMP-dependent pathway. Immature BMDCs from WT mice and IPKO mice were treated with vehicle and different concentrations of cicaprost for 24 h. The cells were then treated with 10 μ g FITC-OVA for 1 h. The expression of CCR7 was detected by flow cytometry. (A) Histogram data show the cells gated for PI⁻ and CD11c⁺. (B) Bar graphs show the percentage of vehicle-treatment. (C) The immature BMDCs from WT were pre-treated with vehicle or Rp-8-Br-cAMPS at 1 μ M or 100 μ M for 30 min, thereafter those cells were treated with cicaprost at 10 nM for 24 h. Values represent the mean $\pm$ SEM of three independent experiments. * P < 0.05 compared with the value of vehicle-treated cells.



DISCUSSION

The most important functions of DCs are the uptake of antigen, migration to regional LNs, and presentation of that antigen to T cells. However, antigen-driven maturation is not essential for DC migration to draining LNs [12]. While the consequence of the migration of immature DCs to draining LNs on T cell function is unclear [25], it is very possible that a decrease in the number of DCs at mucosal surfaces may limit antigen uptake and therefore dampen T cell-driven immune responses. We [11] and others [10] have reported that PGI₂ inhibits the ability of mature DCs to generate antigen-specific allergic responses both in vitro and in vivo, and the data presented in our current study suggest that PGI₂ may have further immunoinhibitory effects by regulating immature DC function. PGI₂ may modulate airway DC functions because PGI₂ concentrations are high in the airway since this eicosanoid is produced primarily by endothelial cells, and vascular structures are in close proximity to airways. In addition, inhaled PGI₂ analogs are approved by the U.S. Food and Drug Administration for the treatment of pulmonary hypertension, and have been reported in the treatment of acute respiratory distress syndrome. Therefore, understanding the effect of PGI₂ on immature DC migration is important for determining the effect that this prostanoid has on antigen-specific T cell activation and the initiation of the adaptive immune response.

In this study, we found that the PGI₂ analog cicaprost decreased the ability of immature DCs to take up antigen in vitro. In addition, our experiments revealed that cicaprost enhanced immature DC podosome dissolution and MMP-9 expression, processes that release the DC from the structural

cells at environmental interfaces allowing for DC migration. Furthermore, we found that cicaprost increased immature DC expression of CCR7, a chemokine receptor that binds CCL19 and CCL21, produced in the T cell zone of LNs in a process enabling DCs to have cognate interactions with naïve T cells that also express CCR7. A functional consequence of the cicaprost-mediated increased CCR7 expression on immature DCs was revealed as there was increased migration of cicaprost-treated DCs to either CCL19 or CCL21 in a transwell assay of cellular migration. Interestingly, cicaprost-treatment not only significantly increased the migration of immature DCs to CCL19 and CCL21, but also increased migration to vehicle in the transwell system, although not to same degree as was seen to the chemokines. This suggests that PGI₂ may also alter cell shape to increase transit through the transwell system. PGI₂ signaling through IP on immature DCs was required for decreased antigen uptake, enhanced podosome dissolution, increased CCR7 expression, increased chemotactic migration toward CCL19 and CCL21, and increased chemokinesis. In addition, we found that immature DCs treated with a PGI₂ analog that was injected into the footpad of a mouse had enhanced migration to the draining popliteal LNs compared to vehicle-treated immature DCs. Finally, cicaprost instillation in the lung induced migration of resident DCs labeled with PKH26 to mediastinal LNs without airway inflammation. These results all suggest that PGI₂ may impede antigen transport to LNs for T cell activation by decreasing antigen uptake directly or reducing the number of DCs at mucosal surfaces for antigen sampling.

We found that cicaprost-treated immature BMDCs highly expressed costimulatory molecules and MHC II. These results are in

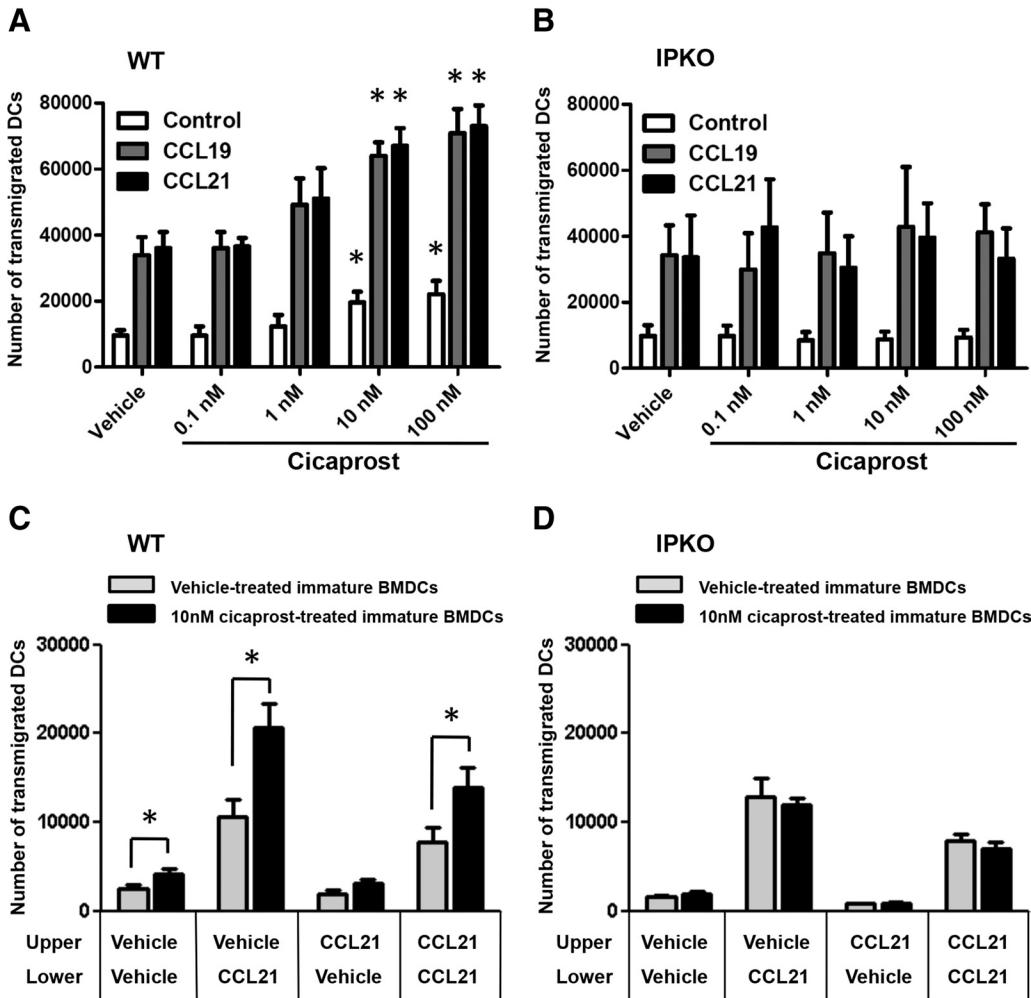


Figure 7. Cicaprost promotes chemotaxis and chemokinesis of immature BMDCs toward CCR7 ligands. (A and B) Immature BMDCs from WT and IPKO mice were treated with 10 nM cicaprost for 24 h. The cells were transferred into the upper chamber of transwell. DC culture medium containing 100 ng CCL19, 100 ng CCL21, or vehicle was added to the lower chamber. After 2 h incubation, the number of BMDCs in the lower chamber was counted. Values represent the mean $\pm$ SEM of four independent experiments. * P < 0.05 compared with the value of vehicle-treated cells. (C and D) Checkerboard analysis was performed to evaluate chemotaxis (directional migration) and chemokinesis (random migration). Values represent the mean $\pm$ SEM of six independent experiments. * P < 0.05 compared with the value of vehicle-treated cells.

contrast to the cicaprost-induced down-regulation of CD40, CD86, and MHC II, we reported in LPS-activated BMDCs [11], thus confirming an important differential effect of cicaprost on immature and mature DCs. We confirmed further that cicaprost-treatment was not activating BMDCs by measuring IL-12p70 and IL-10 in culture supernatant. In contrast to LPS, which induced BMDC production of IL-12p70 and IL-10, cicaprost did not induce expression of these cytokines by BMDCs.

The mechanism by which PGI₂ inhibited immature DC antigen uptake is suggested by studies examining the effect of cAMP in this process. PGI₂-IP signaling increased intracellular cAMP in BMDCs [11, 26] and enhanced cAMP led to activation of PKA. In some cell types, PKA phosphorylated and activated the CREB transcription factor, impairing ERK1,2 signaling [27]. ERK1,2 inhibition or PI3K inhibition decreased FITC-OVA uptake by immature BMDCs. Therefore, cAMP-mediated ERK1,2 inhibition decreased antigen uptake. This is supported by the finding that down-regulation of cAMP production enhanced complement C3a-C3aR signaling-induced FITC-OVA antigen uptake by mouse immature BMDCs [28]. Other PGs also regulate DC antigen uptake, possibly through cAMP mediated actions. PGE₂ which increases cAMP by signaling through EP2 and EP4 [29], inhibited mannose receptor

mediated endocytosis of FITC-dextran by human DCs [30]. However, the concept that cAMP activation inhibits DC antigen uptake may be PG-specific, as PGD₂ increased mannose receptor expression and FITC-dextran uptake by DCs by signaling through DP1, which increases cAMP [31].

Podosomes are specialized adhesion structures that anchor DCs in tissues so that they are present at sites of antigen exposure. Podosome dissolution is a critical first step in high speed DC migration [32]. We found that PGI₂-IP signaling resulted in podosome dissolution in a dose dependent fashion, starting at 10 nM cicaprost. PGs previously have been shown to be essential for podosome dissolution in response to DC activation by Gram-negative bacteria. Gram-negative bacteria matured DCs and induced podosome dissolution through TLR4 signaling and this was blocked by indomethacin treatment. Further studies revealed that LPS-mediated podosome disassembly was a result of DC PGE₂ production [33].

In addition to their effect on podosome dissolution, eicosanoids regulate DC CCR7 and CCR5 surface expression. For instance, PGE₂ and LT₄ enhanced CCR7 expression and reduced CCR5 expression on human monocyte-derived DCs matured with a proinflammatory cytokine cocktail, and facilitated the migration of these cells toward CCL19 or CCL21 [34, 35].

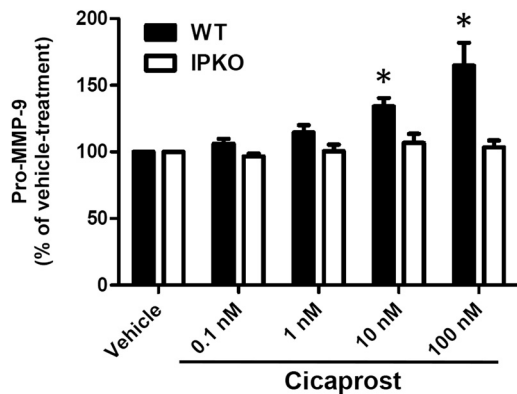


Figure 8. Cicaprost increases pro-MMP-9 expression by immature BMDCs from WT mice, but not IPKO mice. Immature BMDCs from WT mice and IPKO mice were treated with vehicle and different concentrations of cicaprost for 24 h, followed by ELISA for secreted pro-MMP-9. Values represent the mean $\pm$ SEM of four independent experiments. * $P < 0.05$ compared with the value from vehicle-treated cells.

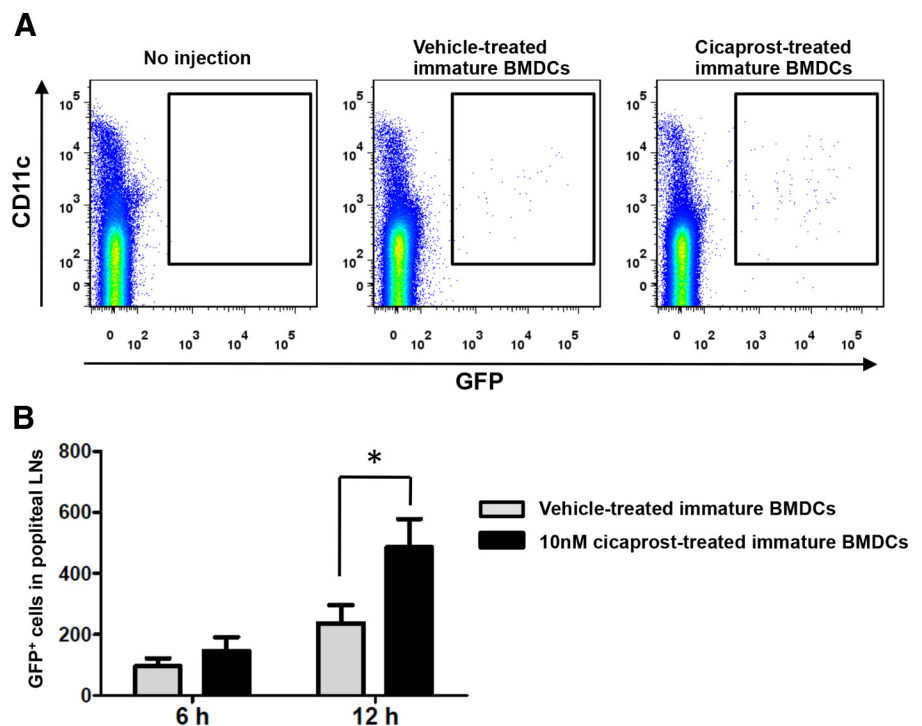
LTB₄-treatment increased mouse immature BMDC chemotaxis toward CCL19 with only a minor effect on chemokinesis [23]. In contrast, PGD₂ signaling through DP1 suppressed LPS-induced CCR7 expression on DCs and reduced migration toward CCL19 [31]. These in vitro results were supported by an in vivo study that revealed that a DP1 agonist inhibited migration to the regional LNs of DCs that had taken up FITC-OVA [36]. Similarly, in a mouse model iloprost administration at the time of FITC-OVA exposure reduced the migration of DCs that had taken up FITC-OVA to draining LNs [10]. While iloprost reduced migration of mature DCs, our studies focused

on PGI₂ analog regulation of immature DCs. Our studies revealed that cicaprost-treatment of immature DCs induced podosome dissolution allowing the cells to leave mucosal surfaces.

MMPs are known for their ability to degrade structural extracellular proteins, such as collagens, elastin, and proteoglycans [15]. PGE₂ signaling through the cAMP, PKA, and ERK pathway induced MMP-9 expression on BMDCs [37, 38], however the effect of PGE₂ depends on the cell type. PGE₂ inhibited MMP-9 expression in the breast cancer cell line MCF-7 and in peritoneal macrophages isolated from women with endometriosis. In addition, EP4-deficient and MMP-9-deficient mice exhibit impaired transmigration of DCs [16, 39]. In this study, we found that cicaprost induced MMP-9 expression on immature BMDCs. This further supports that PGI₂-IP signaling induces DC migration.

To determine the in vivo relevance of our in vitro findings, we used two different models. First, we investigated migration of GFP expressing immature BMDCs from the footpad to popliteal LNs. We found that there was a low level of migration of GFP expressing, immature BMDCs into the popliteal LNs 6 h after the cell injection in both the cicaprost and vehicle-treated BMDCs. However, there was a significant increase in the migration of cicaprost-treated BMDCs to popliteal LNs 12 h after GFP BMDCs were injected in the footpad compared to vehicle-treated BMDCs. GFP expressing immature BMDCs could not be detected in the popliteal LNs 24 h after injection (data not shown). This decrease in survival at 24 h was likely a result of removal of BMDCs from GM-CSF containing culture media. The viability of immature BMDCs without GM-CSF was 70–80% after 24 h and 20–30% after 48 h (data not shown). Immature BMDCs injected in the footpad might have had de-

Figure 9. Cicaprost promotes chemotaxis of immature BMDCs from the footpad to popliteal LNs. Immature BMDCs from the GFP mouse were treated with 10 nM cicaprost or distilled water as vehicle control for 24 h. The cells were injected in the hind-leg footpad. Migrated GFP expressed BMDCs in popliteal LNs were counted by flow cytometry. (A) Migrated GFP expressed cells were determined as PI⁻ (viable), large cells, and GFP⁺. (B) Bar graph shows the number of migrated GFP⁺ cells in popliteal LNs. Values represent the mean $\pm$ SEM of nine mice. * $P < 0.05$ compared between vehicle-treated and cicaprost-treated cells.



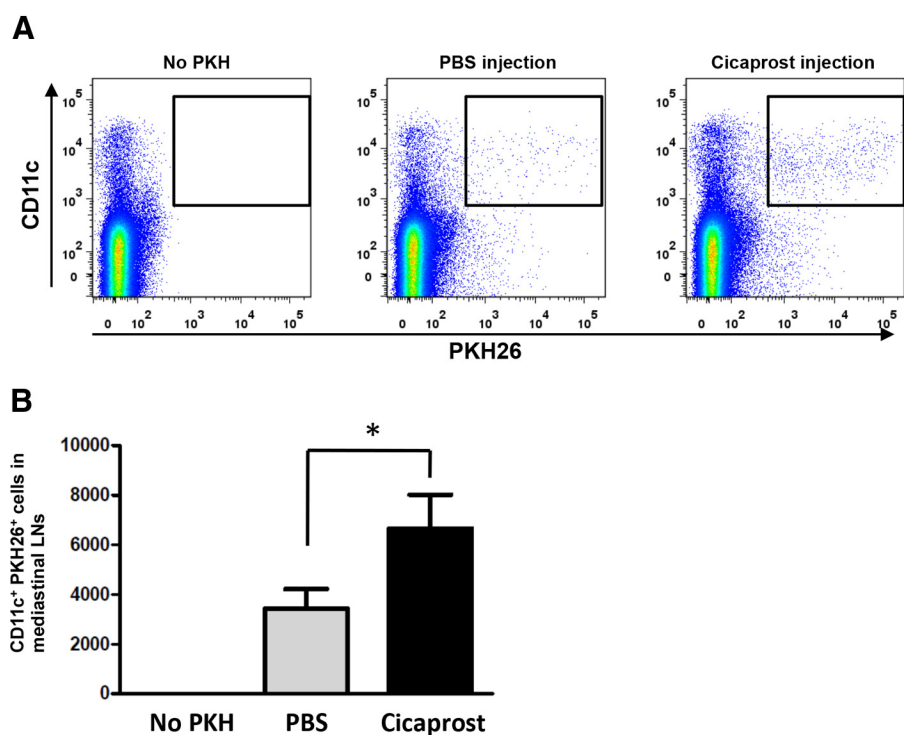


Figure 10. Cicaprost promotes migration of lung immature DCs to mediastinal LNs. WT and IPKO mice were instilled intranasally with 80 μ l of 20 μ M PKH26, 24 h before cicaprost (2.5 μ g/60 μ l) or PBS (60 μ l) instillation. Mediastinal LNs were harvested 12 h after cicaprost or PBS instillation. (A) Migrated lung DCs were determined as PI⁻ (viable), CD11c⁺, and PKH26⁺. (B) Bar graph shows the number of migrated PKH26⁺ DCs in mediastinal LNs. Values represent the mean $\pm$ SEM of nine mice. * P < 0.05 compared between PBS-treated and cicaprost-treated cells.

creased viability since the cells were already cultured for 24 h without GM-CSF, perhaps explaining why immature BMDCs might not be detected in LNs 24 h after footpad injection. In comparison with our migration studies using immature BMDCs, other groups found that LPS-treated mature DCs migrated to the popliteal LNs 24–48 h after injection [40, 41]. LPS significantly induced BMDC viability by increasing the production of cytokines that promote BMDC survival, including GM-CSF [11].

DCs survey epithelial or mucosal surfaces for antigens that are endocytosed or phagocytosed prior to DC migration to LNs for antigen presentation to T cells. However, spontaneous migration of DCs such as Langerhans cells, has been reported in healthy steady-state conditions [42, 43]. These studies suggested that spontaneously migrating, immature DCs might be carrying autologous apoptotic cells from the periphery into the draining LNs [44]. The consequence of this spontaneous migration of DCs is that self-antigens from apoptotic cells could be presented to lymphocytes by immature DCs, thus inducing tolerance instead of immunity against self [45]. This mechanism may also induce tolerance for weakly pathogenic foreign antigens in steady state, however this has not been confirmed. In this study, we detected lung resident DC migration in the steady state in the absence of antigen. CFSE- or FITC-conjugated OVA instillation caused airway inflammation, such as neutrophil accumulation [46]; however, instillation of PKH26 cell linker did not induce airway inflammation and no PKH26-labeled cells were detected in LNs by 4 h after PKH26 instillation [24]. Accordingly, we performed PKH26 instillation before cicaprost injection to assess immature lung resident DC migration in steady state. PKH26⁺ CD11c⁺ cells were detected in mediastinal LNs of WT mice 12 h after PBS injection, how-

ever there was a significant increase in PKH26⁺ CD11c⁺ cells in the LNs following cicaprost administration to the airway. These results suggest that exogenous PGI₂ increases the migration of immature DCs to the LNs.

In conclusion, PGI₂-IP signaling suppressed antigen uptake by immature BMDCs and induced migration of immature BMDCs to draining LNs, thus likely reducing the number of DCs at mucosal epithelial surfaces available for antigen uptake. Consequently, antigen transport is restricted by PGI₂ altering immature DC functions. In addition, PGI₂ may promote self-tolerance by promoting antigen presentation by immature DCs. These are novel concepts by which PGI₂ may decrease antigen specific T cell activation and inhibits adaptive-immune responses. This may have important clinical relevance in patients with pulmonary hypertension who receive inhaled PGI₂.

AUTHORSHIP

S.T. performed and designed experiments, analyzed data, and wrote the manuscript. K.G. and M.M.H. contributed to acquisition of data. W.Z. and D.C.N. advised and contributed to overall development of this study and helped finalize the manuscript. G.A.F. and W.E.L. contributed to mouse study and helped finalize the manuscript. R.S.P. designed and supervised the research, and edited the manuscript.

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KEY WORDS:

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