Inhaled iloprost suppresses the cardinal features of asthma via inhibition of airway dendritic cell function

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Inhalation of iloprost, a stable prostacyclin (PGI2) analog, is a well-accepted and safe treatment for pulmonary arterial hypertension. Although iloprost mainly acts as a vasodilator by binding to the I prostanoid (IP) receptor, recent evidence suggests that signaling via this receptor also has antiinflammatory effects through unclear mechanisms. Here we show in a murine model of asthma that iloprost inhalation suppressed the cardinal features of asthma when given during the priming or challenge phase. As a mechanism of action, iloprost interfered with the function of lung myeloid DCs, critical antigen-presenting cells of the airways. Il oprost treatment inhibited the maturation and migration of lung DCs to the mediastinal LNs, thereby abolishing the induction of an allergen-specific Th2 response in these nodes. The effect of iloprost was DC autonomous, as iloprost-treated DCs no longer induced Th2 differentiation from naive T cells or boosted effector cytokine production in primed Th2 cells. These data should pave the way for a clinical effectiveness study using inhaled iloprost for the treatment of asthma.

Introduction
Asthma is a Th2 lymphocyte–mediated inflammatory airway disease characterized by airway eosinophilia, increased mucus production by goblet cells, and structural remodeling of the airway wall. This leads to variable airway obstruction and bronchial hyperresponsiveness (BHR) to nonspecific stimuli. In the airways of allergen-challenged asthmatics, there is increased production of PGs (1–3). PGs are locally acting autacoids generated by stepwise conversion of arachidonic acid into 2 short-lived intermediates, prostaglandin G and prostaglandin H, through the action of the COX enzymes COX-1 and COX-2 (1, 4, 5). These intermediates can be further metabolized by specific enzymes to a series of products including PGD2, PGE2, PGF2α, prostacyclin (PGI2), and TXA2 (6). PGs have pleiotropic roles in physiologic and pathophysiologic processes, including inflammation and allergic responses (4, 5). In asthma, PGs are generally regarded as proinflammatory molecules, but this view has recently been challenged by work showing that PGE2 can have antiinflammatory and antiasthmatic effects when acting on the E prostanoïd receptor 3 (7) as well as by studies showing that COX-1- and COX-2-deficient mice or mice treated with COX inhibitors had exaggerated inflammatory airway responses and BHR in a murine model of asthma (8–10). These data support the concept that endogenous PGs play a regulatory role in the allergic response with an overall balance favoring suppression of the asthmatic response. As inhibition of COX activity leads to a reduction in a wide range of PGs (e.g., PGD2, PGE2, and PGI2) and downstream metabolites (e.g., the cyclopenteno PG 15-deoxy-Δ2,14-PGJ2), the exact PG or its specific receptor that might be involved in protecting the airways and dampening inflammation during allergen challenge is currently unknown. In this regard, PGI2 may be of great interest because large amounts are produced in a COX-2–dependent manner during IgE-mediated allergic reactions in humans (11, 12) and in mice with Th2-mediated airway inflammation (8). In support of an antiinflammatory effect of PGI2, mice deficient in the PGI2-exclusive I prostanoid (IP) receptor have exaggerated features of acute and chronic experimental asthma, including increased BHR (13, 14). Whether PGI2 would act mainly during sensitization to inhaled allergen or during the allergen challenge phase could not be addressed in IP-deficient mice. In all these models, the precise mechanism of action by which PGI2 might suppress airway inflammation has not to our knowledge been elucidated previously.

Here we used inhaled iloprost, a stable PGI2 analog used clinically as a drug treatment for patients with pulmonary arterial hypertension (15), to study the mechanism of action by which IP triggering suppresses asthma features. We focused particularly on DCs, the most powerful antigen-presenting cells of the immune system. Lung DCs have the unique capacity of stimulating both naive T cells during sensitization and primed Th2 cells during recall responses in the airways (16–20). In the induced absence of DCs, all the cardinal features of asthma such as Th2 cytokine-dependent airway eosinophilia, goblet cell hyperplasia, and BHR disappear (21, 22). Our results show that iloprost suppressed not only the development of asthma when given during the sensitization phase, but also the salient features of experimental asthma during the challenge phase by interfering with the function of these antigen-presenting cells.

Results
Effect of iloprost inhalation during OVA challenge on airway inflammation and BHR. We investigated whether local application of iloprost (in clinically relevant doses, also used for the treatment of pulmo-
nary hypertension) could influence the development of experimental asthma in already sensitized mice. Sensitization to OVA was induced using i.p. injection of OVA/alum (see Methods) on days 0 and 7 and were exposed on days 19–21 to OVA aerosols. Prior to each aerosol, mice received an i.t. injection of vehicle, CAY10449 plus vehicle (OVA/CAY+vehicle/OVA), 0.2 μg iloprost, or CAY10449 plus iloprost. Labels indicate sensitization/treatment/challenge. (A and D) BAL fluid was analyzed by flow cytometry. (B) May-Grunwald-Giemsa staining of lung sections. (C and E) Cytokine production in MLN cells restimulated in vitro for 4 days with OVA. (F) BHR to various doses of i.v. metacholine was assessed for changes in dynamic resistance and lung compliance and BHR to inhaled metacholine for PenH responses was assessed 24 hours after the last antigen exposure were measured. Data are mean ± SEM; n = 8 mice per group. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 1
Local administration of iloprost suppresses asthma features. Mice were sensitized by i.p. injection of OVA/alum (see Methods) on days 0 and 7 and were exposed on days 19–21 to OVA aerosols. Prior to each aerosol, mice received an i.t. injection of vehicle, CAY10449 plus vehicle (OVA/CAY+vehicle/OVA), 0.2 μg iloprost, or CAY10449 plus iloprost. Labels indicate sensitization/treatment/challenge. (A and D) BAL fluid was analyzed by flow cytometry. (B) May-Grunwald-Giemsa staining of lung sections. (C and E) Cytokine production in MLN cells restimulated in vitro for 4 days with OVA. (F) BHR to various doses of i.v. metacholine was assessed for changes in dynamic resistance and lung compliance and BHR to inhaled metacholine for PenH responses was assessed 24 hours after the last antigen exposure were measured. Data are mean ± SEM; n = 8 mice per group. *P < 0.05; **P < 0.01; ***P < 0.001.
did not significantly modify lung function parameters compared with vehicle-treated mice (data not shown).

In response to inhaled metacholine in awake mice, BHR was also assessed by measuring the enhanced pause (PenH) using whole-body plethysmography. BHR to inhaled metacholine was similarly attenuated by iloprost treatment (Figure 1F).

Effect of iloprost inhalation prior to allergen challenge on DC function. As the above data suggested a strong antiinflammatory effect of iloprost, and as it has been previously reported that PGs can affect DC function (24), we addressed the question of whether local iloprost would modify DC function in the airways. Migration of DCs to the MLNs upon allergen encounter is a known function of DCs (25, 26). The total number of DCs in draining MLNs was enumerated 24 hours after the last OVA challenge. The number of DCs was greatly enhanced in OVA-sensitized mice subjected to OVA challenge compared with sham-sensitized mice (Figure 2A). Interestingly, iloprost treatment prior to each allergen challenge markedly decreased the number of DCs (MHCII<sup>high</sup>CD11c<sup>high</sup>) in the draining LNs, and this was accompanied by a strong decrease in the number of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells and CD19<sup>+</sup> B cells (Figure 2B).

In peripheral tissues such as the lung, DCs are found in a so-called immature state, expressing low levels of costimulatory molecules. Maturation of DCs is a key step for the induction and maintenance of allergic airway inflammation and has previously been shown to occur locally in the airways of allergen-challenged mice (22, 27, 28). Maturation is heralded by the expression of costimulatory molecules necessary for optimal T cell expansion and differentiation. As shown in Figure 2C, inhaled iloprost markedly reduced the expression of CD40, CD80, CD83, and CD86 on CD11c<sup>+</sup>MHCII<sup>+</sup> lung DCs of allergen-challenged mice. In the MLNs, there were no significant differences in CD40, CD80, CD83, or CD86 expression between iloprost- and vehicle-treated mice (data not shown). The effects of iloprost were most likely a direct effect on lung DC function, as in bone marrow–derived DCs exposed to OVA in vitro, iloprost significantly decreased the expression of CD40, CD80, CD83, and CD86 (Supplemental Figure 2A). Furthermore, the effect of iloprost on the expression of costimulatory molecules seemed to be DC specific, as treatment of mice with iloprost did not change the expression of CD40, CD80, CD83, and CD86 expression between iloprost- and vehicle-treated mice (data not shown).

Effect of iloprost inhalation on migration of lung DCs to the MLNs. A reduction in DC numbers in the MLNs of iloprost-treated mice could result from a reduction in lung inflammation and concomitant reduction in DC influx into the node (29). Alternatively, iloprost might also directly interfere with the migratory capacity of lung DCs in vivo. To more directly prove an effect of iloprost on the migration of lung DCs, fluorescein isothiocyanate–labeled OVA was injected...
In our initial experiments (Figure 1) we performed allergen challenge phase was caused by alteration of lung DC function, critical for mounting Th2 effector responses in sensitized mice (22). To prove more directly that local iloprost can suppress the function of lung DCs in vivo, we used a model in which Th2 sensitization depends on endogenous lung myeloid DCs (mDCs) (30). Mice were first depleted of tolerogenic plasmacytoid DCs (pDCs) by treatment with anti-Gr-1 Abs, thus leading to priming against inhaled harmless OVA. In Gr-1–treated mice, but not in control isotype-treated mice, OVA inhalation led to strong airway eosinophilia and lymphocytosis as well as goblet cell hyperplasia (Figure 4, A and B). This was accompanied by Th2 cytokine production in the MLNs (Figure 4C). Mice depleted of pDCs that were treated with 0.2 μg iloprost at the time of OVA priming developed no signs of airway inflammation and had 5-fold reduced levels of cytokines in the LN, thus suggesting that iloprost interferes with the potential of lung DC to induce Th2 priming.

However, as iloprost was given via the airways, it would be possible that the inhibitory effects on DCs were indirect and were mediated by structural cells or effects on lymphocytes (8, 31–33). To rule out these indirect effects of iloprost treatment on lung structural cells such as epithelial cells or on lymphocytes, we performed experiments in which mDCs were treated in vitro with iloprost prior to adoptive transfer to the airways of naive mice. As the number of DCs obtained from the lungs of mice was too small to perform large adoptive transfer experiments, we used bone marrow–derived DCs grown in GM-CSF. As previously reported, adoptive i.t. transfer of these OVA-pulsed mDCs leads to Th2 priming and subsequent features of asthma upon OVA aerosol challenge 10 days later, a function that is related to the number of DCs injected (18, 34). In mice receiving unpulsed mDCs, few inflammatory cells accumulated in the BAL fluid and lung tissues after OVA aerosol challenge (Figure 5, A and B). In contrast, in mice receiving OVA-pulsed mDCs, a strong cellular recruitment of lymphocytes and eosinophils occurred in the BAL fluid and peribronchial and perivascular area in the lung, accompanied by goblet cell hyperplasia. The pretreatment of OVA-pulsed mDCs with iloprost ex vivo significantly abolished the potential of these cells to induce eosinophilic airway inflammation and goblet cell hyperplasia (Figure 5B), and this was accompanied by a significant decrease in the level of IL-4, IL-5, and IL-13 in the MLNs, while the concentration of IFN-γ was not significantly changed (data not shown). These data suggest that iloprost treatment of mDCs profoundly inhibits Th2 sensitization. To more directly address this point, we tested the effect of iloprost on DC-driven cytokine production of OVA-specific T cells in vivo (Figure 5C). In order for us to follow primary T cell activation, mice first received a cohort of naive OVA-TCR Tg (DO11.10) T cells, followed 2 days later by an i.t. injection of vehicle-treated OVA-pulsed mDCs, iloprost-treated OVA-pulsed mDCs, or control unpulsed mDCs. DCs were extensively washed to avoid carryover of iloprost in vivo. LN cells (containing OVA-specific T cells) of mice immunized with iloprost-treated OVA-pulsed DCs produced lower levels of the Th2 cytokines IL-4, IL-5, and IL-13 than did cells stimulated with vehicle-treated OVA-pulsed DCs, while the production of IFN-γ and IL-10 was increased. The same effects were seen when iloprost-treated OVA-pulsed mDCs were cocultured in vitro with purified naive (DO11.10) OVA-specific T cells, illustrating that the observed inhibition of Th2 development was inherent to DCs and not due to effects of iloprost on recruitment of T cells in vivo (Supplemental Figure 4).

Effect of in vitro iloprost treatment on the capacity of DCs to activate primed Th2 effector cells. In our initial experiments (Figure 1) we administered iloprost to the airways of already sensitized mice,
leading to a reduction of DC maturation (Figure 2C) and a consequent reduction in airway inflammation. However, these effects could also be due to effects of PGI₂ on T cells, as it has previously been reported that the IP receptor is expressed on Th2 cells (8). To test whether iloprost-exposed DCs would be less efficient in stimulating effector Th2 cells, Th2 effector cells were made in vitro from cultures of DO11.10 OVA-specific T cells in the presence of OVA antigen and polarizing conditions (IL-4, anti–IFN-γ, and anti–IL-12) (22). When restimulated by OVA-pulsed DCs, these Th2 effector cells produced high amounts of IL-4, IL-5, and IL-13, but no IFN-γ (Figure 6). However, treatment of OVA-pulsed DCs with 2.5 μg/ml iloprost prior to culture with Th2 cells strongly inhibited the production of Th2 cytokines.

Discussion

Apart from its dilatory effect on the pulmonary vessels and its anti-thrombotic effects, iloprost, a stable PGI₂ analog, has been shown by several studies to have immune-regulatory effects by altering the production of cytokines and proinflammatory mediators and the expression of adhesion molecules (35–39). Iloprost and/or PGI₂ suppress the function of various immune and inflammatory cells like monocytes/macrophages, lymphocytes, and neutrophils, which has led to the first clinical trials studying its effect as an anti-inflammatory compound (40, 41). Orally administered iloprost can reduce the serum levels of TNF-α and soluble endothelial cell adhesion molecules (like VCAM-1 and ICAM-1) in vivo in patients with rheumatoid arthritis, and this is associated with improvement in the clinical course of the disease (40, 41).

In addition to oral administration, inhalation of aerosolized iloprost is a well-accepted and tolerated therapy for pulmonary arterial hypertension (15). The fact that iloprost can be given by inhalation would make it a feasible treatment option for asthmatic airway inflammation, as inhaled drugs such as corticosteroids and β2-agonist bronchodilators constitute the standard therapy of asthma (42). Our present study demonstrates, for the first time to our knowledge, that inhaled iloprost via selective activation of IP receptors is indeed able to inhibit the salient features of experimental asthma, including Th2 cytokine production, eosinophilic airway inflammation, goblet cell hyperplasia, and BHR.

Several prior studies have suggested that PGI₂ is released in the Airways of allergen-challenged lungs and has an antiinflammatory effect on asthma (3, 8, 11, 12). The strongest evidence comes from studies in IP-deficient mice, which lack the sole receptor for PGI₂ (13, 14). In these mice, features of acute and chronic asthma (i.e., airway remodeling) were severely increased, but it was not reported how a defect in IP signaling led to an increase in inflammation. In addition, Jaffar et al. showed in a Th2 adoptive transfer model of asthma that a selective COX-2 inhibitor (NS-398) given at the time of allergen challenge led to a reduction in lung PGI₂ levels and a consequent increased severity of asthmatic inflammation and BHR (8).

In all the studies reported so far, a clear mechanism by which endogenous PGI₂ reduces inflammation has not been found. PGI₂ has been shown to inhibit allergic mediator release and eosinophil recruitment in humans and experimental animals, but these effects could be indirect (43). Similarly, high levels of PGI₂ are associated with less Th2 lymphocyte recruitment to the lungs, but again, these effects might be indirect due to a reduction in airway inflammation (8). Here we report that iloprost inhalation dramatically altered the function of antigen-presenting DCs. These cells are crucial for both the initiation and the maintenance phases of allergic asthma, as depletion of airway DCs during secondary challenge in sensitized mice abolished all cardinal features of asthma (including airway eosinophilia, goblet cell hyperplasia, and BHR to metacholine), an effect that could be completely restored by adoptive transfer of wild-type DCs (22). DCs are crucial for generating asthmatic inflammation because they can locally interact with Th2 effector cells in the airway wall by providing chemoattract cues for Th2 cells (CCL17 and CCL22) and by delivering MHC and costimulatory signals.
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The periphery of the lung to the MLNs is CCR7 dependent (49, 50). It is well known that the migration of DCs from the lung-derived FITC-OVA carrying DCs to the LNs in naive mice is CCR7 dependent (29), but it could also be a direct effect of iloprost on the potential of lung DCs to migrate to these nodes. The latter concept is supported by our finding that iloprost also suppressed the migration of lung DCs prior to transfer to the airways significantly reduced their potential to induce Th2 effector cytokine production (30, 44). By depleting pDCs, this tolerogenic response is turned into robust Th2 priming by mDCs, and sensitization via the airways alone is a normally tolerogenic event in which pDCs inhibit tolerance to inhaled antigen (20, 30, 44). Inhalation of endotoxin-low OVA is a normally tolerogenic event in which pDCs inhibit the potential of mDCs to prime for effector Th2 cells (30, 44). When properly triggered, mDCs promote Th2 priming while pDCs promote tolerance to inhaled antigen (20, 30, 44). Inhalation of endotoxin-low OVA is a normally tolerogenic event in which pDCs inhibit the potential of mDCs to prime for effector Th2 cells (30, 44).

Iloprost treatment of DCs inhibits their potential to prime for Th2 responses. (A and B) On day 0, mice received an i.t. injection of vehicle-treated OVA-pulsed DCs (vehicle-OVA-DC), iloprost-treated OVA-pulsed DCs, or unpulsed DCs. From days 10–13, all mice were exposed to OVA aerosols. (A) BAL fluid was analyzed by flow cytometry. (B) Hematoxylin and eosin staining of lung sections. (C) On day –2, mice were injected i.v. with OVA-specific naive T cells from DO11.10 mice. On day 0, mice were instilled i.t. with vehicle-treated OVA-pulsed DCs, iloprost-treated OVA-pulsed DCs, or unpulsed DCs. On day 4, LN cells were collected and cultured in 96-well plates for 4 days. (D) Supernatants of bone marrow–derived DCs treated overnight with vehicle or different concentrations of iloprost were collected. The presence of IL-4, IL-5, IL-10, IL-12, IFN-γ were increased. The same conclusions were reached when studying T cell differentiation in vitro. The effects of iloprost treatment of DCs significantly reduced the chemotactic response toward the CCR7 ligand MIP3β in vitro, suggesting a direct effect of this compound on lung DC migration.

Although others suggested that PGI₂ serves an antiasthmatic effect (8, 13, 14), it is unclear at present whether PGI₂ and IP signaling interfere with the sensitization phase of allergic asthma, as the IP-deficient mice had a constitutive deletion affecting both sensitization and challenge phases (13, 14) and the studies using COX-2 inhibitors employed a passive Th2 transfer model of asthma, in which the sensitization phase is bypassed (8). In allergic sensitization, when antigen is recognized for the first time by the cells of the pulmonary immune system, DCs play a crucial role. When properly triggered, mDCs promote Th2 priming while pDCs promote tolerance to inhaled antigen (20, 30, 44). Inhalation of endotoxin-low OVA is a normally tolerogenic event in which pDCs inhibit the potential of mDCs to prime for effector Th2 cells (30, 44). By depleting pDCs, this tolerogenic response is turned into robust Th2 priming by mDCs, and sensitization via the airways was observed. Under these conditions, iloprost completely abolished the development of Th2 effector cells; consequently, asthma did not develop upon repeated OVA challenge. The most likely explanation was a direct inhibitory effect of iloprost on lung DCs and not an indirect effect on epithelial cells. This notion is supported by the finding that the ex vivo pretreatment of OVA-pulsed mDCs prior to transfer to the airways significantly reduced their potential to induce Th2 priming. These effects were due to defective priming, as DO11.10 Th2 cell differentiation in the MLNs following i.t. injection of mDCs was severely impaired. Strikingly, however, the levels of the immunoregulatory cytokine IL-10 and the Th1 cytokine IFN-γ were increased. The same conclusions were reached when studying T cell differentiation in vitro. The effects of...
Iloprost treatment on T cell differentiation could be due to reduced costimulatory molecule expression by DCs (Figure 2C), reduced migration of DCs to the LNs (Figure 3), or altered cytokine production following iloprost exposure. In bone marrow–derived DCs, iloprost indeed inhibited the production of IL-12 and TNF-α, while it increased the release of IL-10 (Figure 5D), as previously shown (38). How this could affect Th2 priming is unclear at present, but clearly TNF-α might be required for optimal induction of a proinflammatory state (51).

In conclusion, the present study demonstrates for the first time to our knowledge that iloprost inhibits Th2-mediated cardinal features of asthma by altering the function of lung DCs. As iloprost inhalation is a well-tolerated and safe treatment for pulmonary hypertension, our findings add a new therapeutic effect in experimental asthma should pave the way for a study addressing the effectiveness of this compound in humans with asthma. This paper validates the concept that targeting airway DC function is a powerful method to treat asthma.

Methods

**Mice.** BALB/c mice (6–8 weeks old) were purchased from Harlan. OVA-TCR Tg mice (DO11.10) on a BALB/c background were bred at the Erasmus Medical Center. All experimental protocols were approved by the animal ethics committee at the Erasmus Medical Center.

**OVA/alum model of asthma.** Mice were sensitized to OVA by i.p. injection of OVA/alum (10 μg OVA grade V adsorbed to 1 mg aluminum hydroxide; Sigma-Aldrich) on days 0 and 7 and were challenged with OVA aerosols (grade III) on days 17–19, using a jet nebulizer delivering 1% OVA in PBS for 30 minutes (21). Thirty minutes before each OVA exposure, mice were anesthetized with avertin and given an i.t. injection of control vehicle or 2.5 μg/ml iloprost added 30 minutes before the addition of OVA. As a control, DCs were incubated with PBS containing the vehicle. After antigen pulsing, nonadherent DCs were collected, washed to remove free OVA or iloprost, and resuspended in PBS at a concentration of 12.5 × 10^6 cells/ml.

For in vivo experiments, BALB/c mice were anesthetized on day 0 with avertin (2% v/v in PBS), and 1 × 10^6 vehicle-treated DCs, OVA-pulsed DCs, or iloprost-treated OVA-pulsed DCs were instilled through the opening vocal cords as described previously (18). On days 10–12, mice were exposed to a 30-minute OVA aerosol. Mice were sacrificed 24 hours after the last aerosol.

**Flow cytometry and sorting.** After counting and washing, BAL cells were stained for 30 minutes with FITC-labeled anti-I-Ad/I-Ed (macrophages/DCs), PE-labeled anti-CCR3 (eosinophils), Cy-chrome-labeled anti-CD3 and anti-CD19 (lymphocytes), and allophycocyanin-labeled (APC-labeled) anti-CD11c (macrophages/DCs) in PBS containing 0.5% BSA and 0.01% sodium azide. Differential cell counts were analyzed by flow cytometry, as previously described (52).

For determination of lymphocytes and DC number in the MLNs, MLN cells were stained for T cell subsets (FITC-labeled anti-CD3, PE-labeled anti-CD4, Cy-chrome-labeled anti-CD8), B cells (PE-labeled anti-CD19) or DCs (FITC-labeled anti-MHCII, APC-labeled anti-CD11c). Absolute cell number was calculated by multiplying the total leukocyte number by the percentage of each population of interest.

For analysis of DC maturation, bone marrow, lung, or LN cell suspensions were stained with FITC-labeled anti-I-Ad/L-Ed, PE-labeled anti-CD40, anti-CD80, anti-CD83, and anti-CD86; and APC-labeled anti-CD11c. Absolute cell number was determined by counting the total leukocyte number by the percentage of each population of interest.

In all experiments, dead cells were excluded from analysis using propidium iodide. Analysis was performed on a FacsCalibur flow cytometer (BD Biosciences) using CellQuest version 3.3 (BD Biosciences) and FlowJo version 6.4.7 (TreeStar Inc.) software.

**Determination of BHR.** Twenty-four hours after the last OVA aerosol challenge, nonspecific airway responsiveness was measured by exposing awake mice to aerosolized PBS to set a baseline value, followed by increasing concentrations of aerosolized metacholine (1.5625, 3.125, 6.25, 12.5, and 25 mg/ml) in PBS for 3 minutes; Sigma-Aldrich) using ultrasonic nebulizers. PenH values were measured for 3 minutes after each metacholine aerosol using a whole-body plethysmograph (Buxco Electronics). The average PenH values were expressed for each metacholine concentration as the percentage increase over baseline PenH values (53).
For invasive measurement of dynamic resistance and compliance, mice were anesthetized with urethane, paralyzed using tubocurarine, tracheotomized, and intubated with an 18-gauge catheter, followed by mechanical ventilation with a Flexivent apparatus (Scribner). Respiratory frequency was set at 120 breaths per min with a tidal volume of 0.2 ml and a positive end-expiratory pressure of 2 ml H2O. Increasing concentrations of metacholine (0–600 μg/ml) were administered via the jugular vein. Dynamic resistance and compliance was recorded after a standardized inhalation maneuver given every 10 seconds for 2 minutes (54–56). Baseline resistance was restored before administering the subsequent doses of metacholine.

Effect of iloprost on the activation of OVA-specific naive T cells in a primary immune response. Because the frequency of OVA-specific T cells is very low in naive animals, the primary activation of a naive T cell is difficult to detect. To avoid this problem, naive T cells purified from DO11.10 mice were adoptively transferred into BALB/c mice. Briefly, LN and spleen were collected from DO11.10 mice and homogenized, and 10^6 live cells were injected i.v. in the lateral tail vein of BALB/c mice (day –2). On day 0, the mice received an i.t. injection of OVA-pulsed DCs, iloprost-treated OVA-pulsed DCs, or control unpulsed DCs. On day 4, MLNs were collected and homogenized, and LN cells (200,000 cells/well in triplicate) were resuspended in RPMI 1640 containing 5% FCS and antibiotics and placed in 96-well plates. Four days later, supernatants were harvested and analyzed for the presence of IL-4, IL-5, IL-10, IL-12, and IFN-γ.

Activation of OVA-specific memory Th2 cells by mDCs. Naive CD4+ T cells (1 x 10^5) were purified from unmanipulated DO11.10 mice and were first differentiated for 7 days into effector Th2 cells in the presence of IL-4, anti–IFN-γ, and anti–IL-12, as previously described (22). After washing, these effector Th2 cells were cocultured with bone marrow–derived DCs (1 x 10^5), which were pretreated with iloprost or vehicle alone in round-bottomed 96-well tissue culture plates. After 4 days, supernatants were harvested and analyzed for the presence of IFN-γ, IL-4, IL-5, and IL-13 cytokines by ELISA. Cytokine measurements. To measure cytokine levels, MLNs were plated in round-bottomed 96-well plates (1 x 10^6 cells/ml) and restimulated with OVA (10 μg/ml) for 4 days. The presence of IL-4, IL-5, IL-13, IL-10, TNF-α, and IFN-γ was assayed on supernatants by ELISA (BD).

Statistics. For all experiments, the difference between groups was calculated using the Mann-Whitney U test for unpaired data. A P value less than 0.05 was considered to be significant. Groups of mice consisted of at least 8 mice. Experiments were repeated at least 3 times.

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