Activation of the D prostanoid 1 receptor suppresses asthma by modulation of lung dendritic cell function and induction of regulatory T cells

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Prostaglandins (PGs) can enhance or suppress inflammation by acting on different receptors expressed by hematopoietic and nonhematopoietic cells. Prostaglandin D_2 binds to the D prostanoid (DP)1 and DP2 receptor and is seen as a critical mediator of asthma causing vasodilation, bronchoconstriction, and inflammatory cell influx. Here we show that inhalation of a selective DP1 agonist suppresses the cardinal features of asthma by targeting the function of lung dendritic cells (DCs). In mice treated with DP1 agonist or receiving DP1 agonist–treated DCs, there was an increase in Foxp3$^+$ CD4$^+$ regulatory T cells that suppressed inflammation in an interleukin 10–dependent way. These effects of DP1 agonist on DCs were mediated by cyclic AMP–dependent protein kinase A. We furthermore show that activation of DP1 by an endogenous ligand inhibits airway inflammation as chimeric mice with selective hematopoietic loss of DP1 had strongly enhanced airway inflammation and antigen-pulsed DCs lacking DP1 were better at inducing airway T helper 2 responses in the lung. Triggering DP1 on DCs is an important mechanism to induce regulatory T cells and to control the extent of airway inflammation. This pathway could be exploited to design novel treatments for asthma.

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Abbreviations used: BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; COX, cyclooxygenase; DP, D prostanoid; EP, E prostanoid; i.t., intratracheal; mDC, myeloid DC; MHC, metacholine; MLN, mediastinal LN; pDC, plasmacytoid DC; PenH, enhanced pause; PG, prostaglandin; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PKA, protein kinase A.

Asthma is a Th2 lymphocyte-mediated inflammatory disease characterized by airway eosinophilia and remodeling, goblet cell hyperplasia, and bronchial hyperresponsiveness. The prostaglandin (PG) series of arachidonic acid metabolites are potent regulators of inflammation. Mast cell–derived prostaglandin D_2 (PGD_2) is a proinflammatory mediator causing vasodilation, bronchoconstriction, and chemoattraction of eosinophils and Th2 cells (1, 2). It is found in increased levels in the bronchoalveolar lavage (BAL) fluid of atopic asthmatics and mice with experimentally induced asthma (3, 4). Mice overexpressing the lipocalin-type PGD synthase in the lungs demonstrated enhanced features of asthma (5), whereas reduction of PGD_2 synthesis by uteroglobin was associated with reduced allergic inflammation (3).

The paradigm that PGs promote inflammation was challenged by experiments showing that genetic deficiency of the cyclooxygenase (COX)-1 and -2 enzymes unexpectedly led to strongly enhanced features of asthma through deficient production of PGD_2 and/or prostaglandin E_2 (PGE_2) (6). Similarly, nonsteroidal antiinflammatory drugs such as aspirin or COX inhibitors that block PG synthesis are largely ineffective in the treatment of asthma and can even exacerbate it (7). The exact role of a specific prostanoid in the inflammatory response is often ambiguous. By acting on different subtype receptors on both hematopoietic and nonhematopoietic cells, PGs can exert opposing pro- or antiinflammatory effects (8–10). For example, PGE_2 promotes or suppresses Th2 responses by acting on the E prostanoid (EP)4 receptor or EP3 receptor, respectively (11).

Whether PGD_2 could also have antiinflammatory effects through selective usage of the D prostanoid (DP)1 or DP2 receptor is currently unknown (9). Mice constitutively deficient in

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the DP1 receptor had severely impaired features of asthma (12). The proasthmatic effects of PGD_2 could also be mediated by DP2 (also known as CRTH2), mediating direct chemoattraction of eosinophils, basophils, and Th2 cells and bronchial hyperreactivity (2, 13–15), although again this issue is controversial (16). PGD_2 can also recruit inflammatory cells indirectly by inducing Th2-selective chemokines (e.g., monocyte-derived chemokine CCL22) in bronchial epithelial cells, although the precise DP receptor involved is unknown (17).

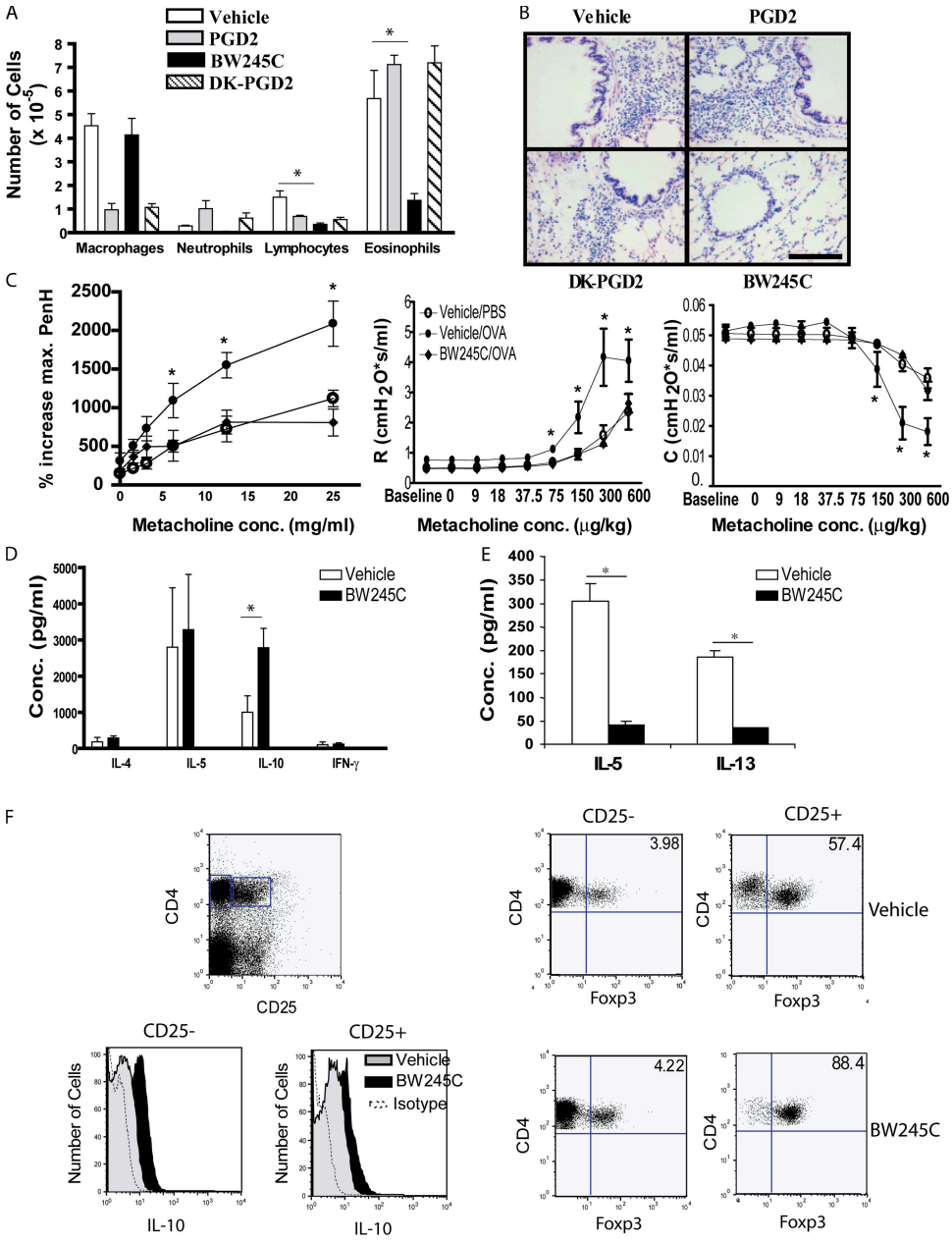

Figure 1. Administration of the DP1 agonist BW245C suppresses asthma features. Mice were sensitized by an i.p. injection of OVA-alum on days 0 and 7. On days 17–20, mice were exposed to OVA aerosols. 30 min before each aerosol, mice received an i.t. injection of Vehicle (white), PGD_2 (grey), BW245C (black), or DK-PGD_2 (hatched). (A) BAL fluid was analyzed by flow cytometry. *, $P < 0.05$. (B) May Grunwald Giemsa staining of lung sections. (C) BW245C inhibits the induction of BHR. Airway obstruction to increasing doses of inhaled metacholine for PenH responses (% increase from saline) or to i.v metacholine for changes in resistance (R) and lung compliance (C) were measured 24 h after the last antigen exposure. *, $P < 0.05$. (D) Cytokine measurements of MLN cells restimulated in vitro for 4 d with OVA. *, $P < 0.05$. (E) BAL supernatants were collected and assayed for the presence of IL-5 and IL-13. (F) The percentage of $Foxp3^+$ cells in $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells in MLNs was determined by flow cytometry. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were also stained intracellularly for the presence of IL-10. $n = 8$ mice/group. Bar, 85 μm. conc., concentration.

We now show that treatment with a DP1 agonist potently inhibits the cardinal features of asthma by altering the function of lung DCs. These cells are derived from the hematopoietic system and play a critical role in asthma pathogenesis by inducing Th2 sensitization to inhaled allergen and by mounting effector Th2 responses in already established airway inflammation. (18–21). Although our data seem to contradict the observed reduction in asthma in constitutive $DP1^{-/-}$ mice, we furthermore show that chimeric mice with hematopoietic deletion of DP1 had strongly enhanced eosinophilic airway inflammation through enhanced DC function. This antiinflammatory DP1 pathway might be exploited for the design of new asthma therapeutics.

RESULTS

Treatment with a selective DP1 agonist during OVA challenge suppresses asthma

To study the exact contribution of the two DP receptors in asthma, we administered PGD_2, the selective DP1 agonist BW245C, or the selective DP2 agonist DK-PGD_2 at the time of allergen challenge in OVA-sensitized BALB/c mice. Mice receiving vehicle before each OVA aerosol challenge developed BAL fluid and peribronchial lung tissue eosinophilia and lymphocytosis, accompanied by Th2 cytokine production in the mediastinal LNs (MLNs) (Fig. 1, A and B). BW245C treatment strongly suppressed inflammation, whereas PGD_2 or 13,14-dihydro-15-keto–PGD_2 was without effect. Bronchial hyperresponsiveness (BHR) to nonspecific stimuli like metacholine is one of the defining symptoms of allergic asthma. BHR was first assessed by measuring the enhanced pause (PenH) using whole body plethysmograpy in response to inhaled metacholine in awake mice. BHR to inhaled metacholine was attenuated by BW245C treatment (Fig. 1 C). As shown in Fig. 1 C, the allergen challenge of OVA-sensitized mice induced a significant change ($P < 0.05$) in responsiveness to i.v. metacholine compared with sham-sensitized mice, as measured 24 h after the last OVA aerosol challenge by invasive measurement of dynamic resistance (R) and compliance (C) in mechanically ventilated mice. Inhalation of BW245C before each allergen challenge markedly attenuated the OVA-induced change in metacholine responsiveness. Strikingly, in BW245C-treated mice the levels of the immunoregulatory cytokine IL-10 in MLN cells were higher, whereas the levels of IL-4, IL-5, and IFN-γ were identical compared with vehicle-treated mice (Fig. 1 D). Strikingly, the levels of Th2 cytokines measured in the BAL of BW245C-treated mice were reduced compared with mice treated with the vehicle (Fig. 1 E), suggesting a local effect of BW245C on Th2 cytokine production. Intracellular cytokine staining on MLN cells revealed that IL-10 production was detectable in $CD4^+$ cells. When gated on $CD4^+CD25^+$ and $CD4^+CD25^-$ MLN T cells, both subsets contained IL-10 and levels were consistently higher in mice treated with BW245C compared with vehicle (Fig. 1 F). Within $CD4^+CD25^+$ cells, a considerable proportion of cells expressed the regulatory T (T reg) cell–associated transcription

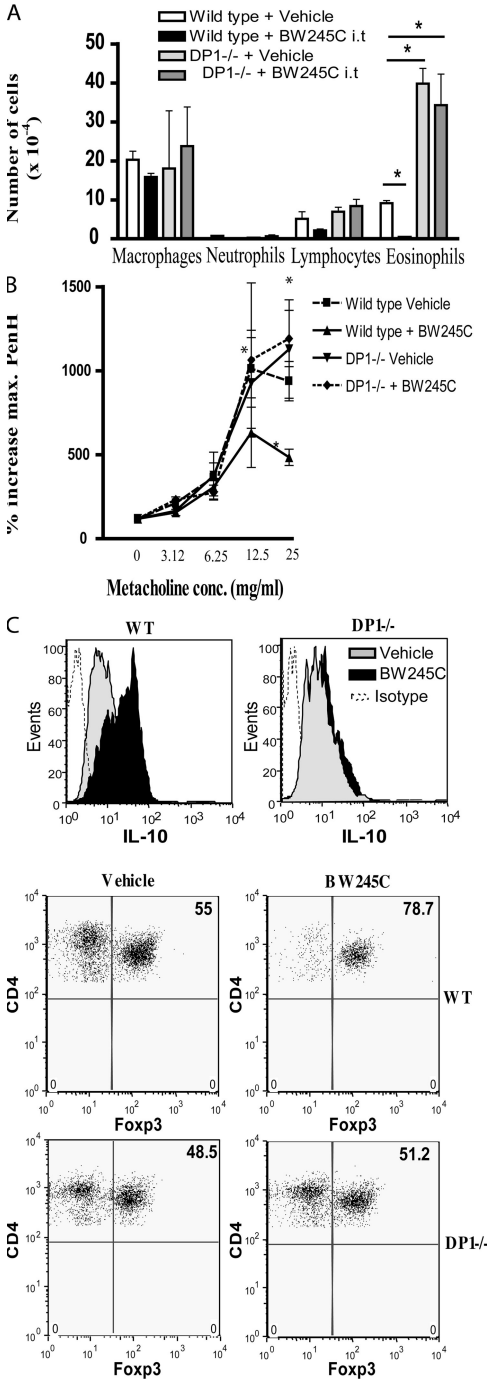

Figure 2. The antiinflammatory effect of BW245C is dependent on DP1 receptor expression on BM-derived cells. WT BM chimeras and DP1 knockout mice BM chimeras were sensitized and challenged as in Fig. 1. (A) BAL fluid differential counts. (B) Airway obstruction to increasing doses of inhaled metacholine was measured as increase in PenH values (% increase from saline) 24 h after the last antigen exposure. (C) $CD4^+CD25^+$ T cells from MLNs of WT or DP1 knockout mice BM chimeras were stained intracellularly for the presence of IL-10. The percentage of $Foxp3^+$ cells in $CD4^+CD25^+$ T cells was determined by flow cytometry. Data are shown as mean ± SEM. *, $P < 0.05$. $n = 8$ mice per group. conc., concentration.

factor Foxp3, and this percentage was strongly enhanced in BW245C-treated mice.

To exclude nonspecific effects of BW245C treatment on another prostanoid receptor, we made chimeric mice in which the DP1 receptor is absent on hematopoietic cells by reconstituting lethally irradiated WT C57BL/6 mice with $DP1^{-/-}$ C57BL/6 BM cells or as a control with WT cells. In chimeric $DP1^{-/-}$ mice, there was a remarkable fourfold increase in the degree of eosinophilic airway inflammation compared with WT chimeric mice (Fig. 2 A). Treatment with BW245C had the same antiinflammatory effects in the WT C57BL/6 strain as in BALB/c mice but did not suppress inflammation or BHR in $DP1^{-/-}$ chimeric mice (Fig. 2, A and B). Intracellular cytokine staining on MLN cells showed IL-10 production in $CD4^+CD25^+$ cells of WT and $DP1^{-/-}$ BM chimeric mice. IL-10 levels were consistently higher in cells of WT BM chimeric mice treated with BW245C compared with vehicle (Fig. 2 C). Moreover, the percentage of $Foxp3^+$ cells was strongly enhanced in BW245C-treated WT chimeric mice. No increased expression of IL-10 and Foxp3 was observed when $DP1^{-/-}$ chimeric mice were treated with BW245C compared with vehicle (Fig. 2 C).

Treatment with DP1 agonist during challenge alters DC maturation

As PGD_2 and BW245C alter some functions of DCs in vivo (22), and ongoing allergic responses depend on DCs (21), we next studied the treatment effect on DC maturation. In BW245C-treated WT chimeric mice, there was a significantly reduced expression of the maturation marker CD86, CD40, and MHC II on lung DCs of OVA-challenged mice (Fig. 3 A). Interestingly, these effects of BW245C were abolished in $DP1^{-/-}$ chimeric mice, except for CD40 whose expression was still reduced compared with vehicle-treated mice (Fig. 3 B). Intracellular IL-10 levels were clearly detectable in lung-derived DCs from OVA-exposed mice (23), but BW245C treatment did not lead to an increased expression (Fig. 3 D).

To more directly study the effect on DC maturation, we treated BM DCs in vitro with BW245C. Our commercially

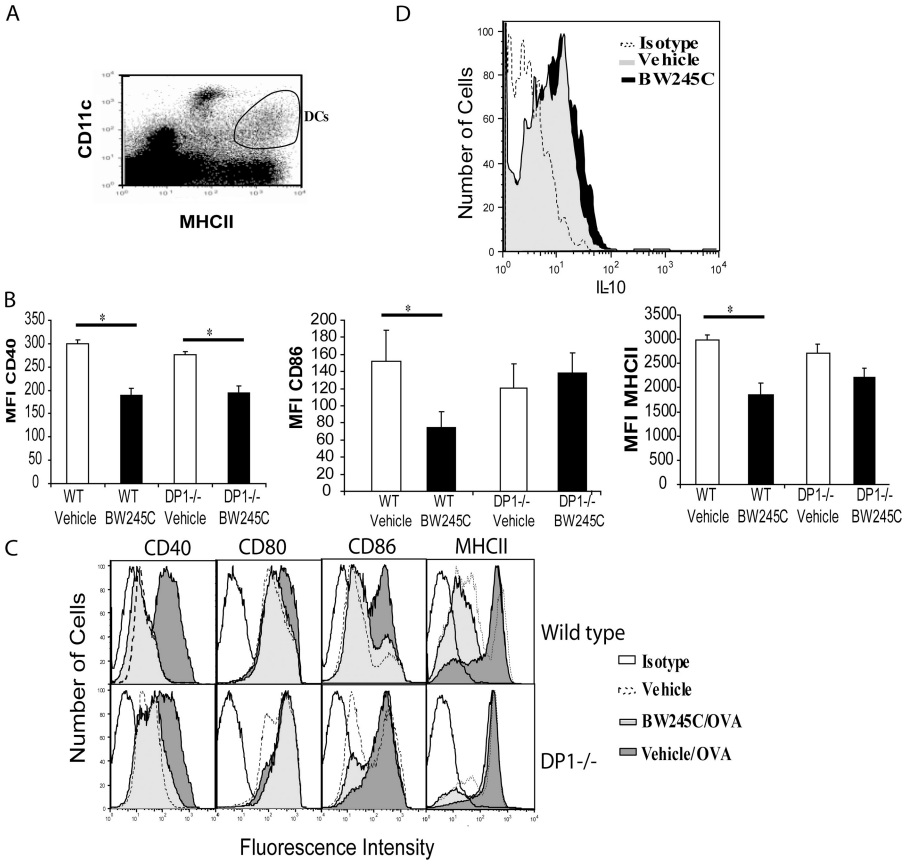

Figure 3. DP-1 activation affects the phenotype of DCs. Mice were sensitized and challenged and treated with DP agonists as in Fig. 1. (A) Single cell suspension was prepared from the lungs of WT or $DP1^{-/-}$ BM chimeric mice, and $CD11c^+MHCII^{hi}$ lung DCs were analyzed for their expression of CD40, CD86, and MHC II. Data are shown as mean fluorescence intensity ± SEM. *, $P < 0.05$. MFI, mean fluorescence intensity (B) BMDCs from WT or DP-1 knockout mice were pulsed or not with 100 μg/ml of OVA overnight. DCs were also treated for 30 min with vehicle (Vehicle/OVA-DCs) or BW245C before addition of OVA (BW245C/OVA-DCs). DCs were collected, and the expression of CD40, CD80, CD86, and MHC II was analyzed by flow cytometry. (C) Analysis of intracellular IL-10 staining in $CD11c^+MHCII^{hi}$ DCs of OVA-challenged mice treated or not with BW245C.

available batch of OVA contains trace amounts of LPS sufficient to induce some degree of DC activation (up-regulation of CD40 and CD86) (Fig. 3 C). The treatment of OVA-DCs with BW245C strongly suppressed the increase in CD86 and MHC II expression, an effect that was not present when DCs were derived from DP1$^{-/-}$ donors. Again the suppression of CD40 was DP1 independent. In DP1$^{-/-}$ DCs, the levels of OVA-induced CD40, CD86, and MHC II were consistently high and unimodal, whereas in WT DCs some cells were still in an immature CD86^{low} MHC II^{low} state.

DP1 ligation inhibits the induction of airway Th2 responses by lung myeloid DCs

To prove more directly that DP1 ligation can suppress the function of lung DCs in vivo, we used a model in which Th2 sensitization depends on endogenous lung myeloid (m)DCs (20). Mice were first depleted of tolerogenic plasmacytoid (p)DCs by treatment with anti–Gr-1 antibodies, thus leading to priming to inhaled harmless OVA. In Gr-1–treated mice, but not in control mice, OVA inhalation led to strong airway eosinophilia and lymphocytosis (Fig. 4, A and B). Mice treated with BW245C at the time of OVA priming no longer developed signs of airway inflammation. To rule out any indirect effects of BW245C on lung structural cells, we performed experiments in which BM-derived mDCs were treated in vitro with BW245C before adoptive transfer to the airways in vivo, a protocol leading to Th2 sensitization and features of asthma in BALB/c and C57BL/6 mice (19 and Fig. 5, A and B). The pretreatment of OVA-DCs with BW245C inhibited their capacity to prime for airway inflammation and Th2 responses in the MLN in a DP1-dependent way (Fig. 5 B). BW245C-pretreated OVA-DCs induced higher levels of IL-10 and IFN-γ compared with vehicle. Strikingly, OVA-DCs from DP1$^{-/-}$ mice were consistently more effective at inducing airway inflammation compared with WT DCs and also induced the highest levels of IL-5 in the LN. Analogous to treatment in vivo (Fig. 1 E), pretreatment of WT OVA-DCs with BW245C led to an increase in the percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells and IL-10$^+$ T cells in the MLNs; the latter effects were dependent on DP1 (Fig. 5 C).

DCs exposed to DP1 agonist in vitro induce the differentiation of T reg cells with antiinflammatory potential

The inhibitory effects of BW245C on induction of asthma suggested that DP1 ligation profoundly altered T cell polarization. To gain more information on this process, we next studied DC-driven T cell responses of OVA-specific (DO11.10) T cells in vitro. BW245C-treated OVA-DCs induced a lower proliferation and lower production of IL-5 in DO11.10 T cells compared with vehicle-treated OVA-DCs (Fig. 6, A and B), whereas the levels of the immunoregulatory cytokine IL-10 were strongly increased. The levels of IFN-γ induced by OVA-DC were unaffected by BW245C. Moreover, the percentage of CD4$^+$25$^+$Foxp3$^+$ cells was

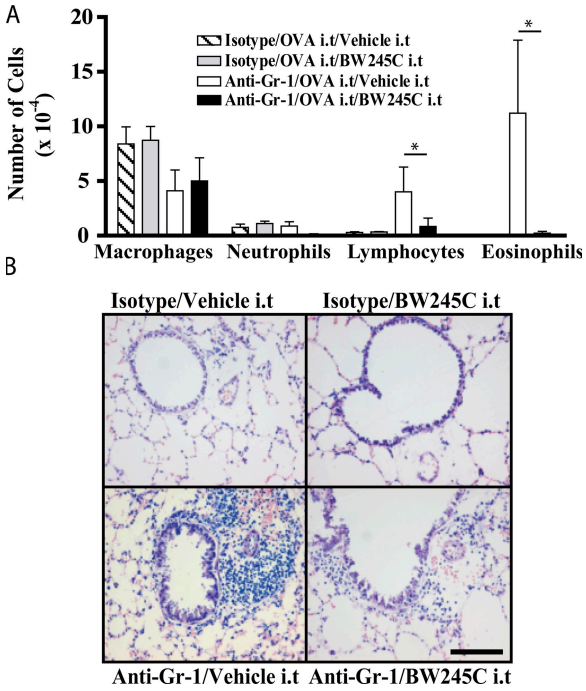

Figure 4. Administration of BW245C affects lung myeloid DCs and inhibits airway inflammation. On day 0, mice received an i.t. injection of OVA in the presence or in the absence of BW245C. From days −1 to 2, mice were injected i.p. with anti–Gr-1 antibodies to deplete pDCs or isotype control antibodies. 10 d later, mice were exposed to three OVA aerosols. (A) BAL fluid was analyzed by flow cytometry. Data are shown as mean ± SEM. (B) May Grunwald Giemsa staining of lung sections. Bar, 85 μm .

increased when T cells were cocultured with BW245C/OVA-DCs compared with cells cocultured with vehicle/OVA-DCs (Fig. 6 C). As we found high levels of IL-10 induction and a higher number of Foxp3$^+$ T cells with BW245C-treated OVA-DCs in vitro and in vivo (Fig. 1 D and Fig. 5 C) associated with a reduced proliferation of naive T cells, and we found increased numbers of Foxp3$^+$CD25$^+$ cells in vivo (Fig. 1 F and Fig. 5 C), we investigated whether BW245C-treated OVA-DCs induce the differentiation of T reg cells. As shown in Fig. 6 C, when OVA-DC–stimulated DO11.10 T cells were added to splenocytes and fresh naive DO11.10 T cells, OVA-specific T cell proliferation was stronger because of the presence of effector cells. However, BW245C/OVA-DC–stimulated T cells reduced the proliferation of fresh naive OVA-specific T cells in response to OVA-pulsed splenocytes.

To prove DP1 ligation on DCs could also induce the differentiation T cells with regulatory properties in vivo, CD4$^+$ T cells obtained from the MLNs of mice injected with OVA-DCs or with BW245C/OVA-DCs were transferred into OVA-alum–sensitized recipients. When CD4$^+$ T cells from OVA-DC–injected mice were transferred, the number of eosinophils in the BAL fluid was substantially increased compared with mice that did not receive T cells (Fig. 6 E).

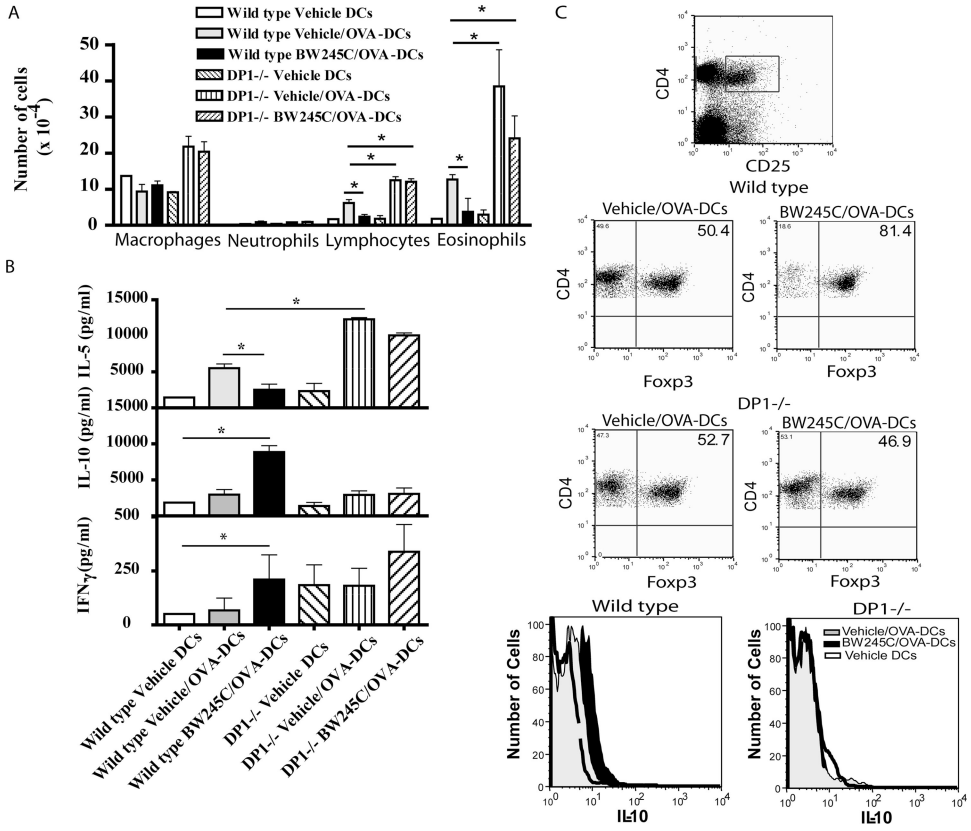

Figure 5. BW245C treatment of DCs inhibits BAL eosinophilia. On day 0, mice received an i.t. injection of vehicle/OVA-DCs, BW245C/OVA-DCs, or vehicle DCs obtained from WT or DP1 knockout mice. From days 10–13, mice were exposed to OVA aerosols. (A) BAL fluid differential count. (B) MLN cells restimulated in vitro for 4 d with OVA and cytokine levels measured in the supernatant. (C) Foxp3 and IL-10 expression in $CD4^+CD25^+$ cells obtained from the MLNs of the animals. Data are shown as mean ± SEM. *, $P < 0.05$. $n = 8$ mice per group.

The adoptive transfer of $CD4^+$ T cells obtained from mice injected with BW245C/OVA-DCs could, however, partially inhibit BAL eosinophilia, suggesting the presence of T reg cell activity.

Some T reg cells suppress inflammation through the anti-inflammatory cytokine IL-10. To address if enhanced IL-10 secretion (Fig. 5 B) was responsible for the absence of asthmatic inflammation in BW245C/OVA-DC–immunized mice, some mice received an i.p injection of blocking anti–IL-10R antibodies 1 d before the OVA aerosol exposure period. The injection of anti–IL-10R antibodies to BW245C/OVA-DC–immunized mice restored airway eosinophilia (Fig. 6 F) to levels observed in OVA-DC–immunized mice. To directly prove that recipient IL-10 was necessary for the observed inhibitory effect, the next approach was to transfer BW245C-treated or untreated OVA-DCs into either WT or $IL\text{-}10^{-/-}$ C57BL/6 recipients. BW245C/OVA-DCs failed to induce airway eosinophilia and lymphocytosis (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20061196) and IL-5 cytokine production in MLN in WT recipient mice, but on the contrary strongly boosted airway inflammation when injected into $IL\text{-}10^{-/-}$ recipient mice (not depicted). The production of IL-5 by MLN T cells of $IL\text{-}10^{-/-}$ recipients was considerably increased compared with WT recipient mice but only when OVA-DCs were pretreated with BW245C (unpublished data).

DP1 ligation of DCs alters DC function through cAMP-dependent protein kinase A activation

The seven transmembrane-spanning G protein–coupled DP1 receptor is known to stimulate adenyl cyclase through Gαs proteins. In inflammatory and immune cells an accumulation of cAMP is generally associated with suppression of effector functions, partially through activation of the cAMP-dependent protein kinase A (PKA) pathway. As shown in Fig. 6 G, BW245C pretreatment of OVA-pulsed DCs was no longer able to induce the production of IL-10 in naive DO11.10 T cells in the presence of a PKA antagonist Rp-8-Br-cAMP (5 μM), whereas exposure of OVA-pulsed DCs to a stable cell-permeable cAMP analogue 8-Br-cAMP (1 μM) mimicked the effects of BW245C in inducing high levels of IL-10 production in T cells. Similarly, the effects of BW245C in inhibiting DC maturation (CD40 up-regulation) of BM-derived DCs were abolished in the presence of a PKA inhibitor and mimicked by stable cAMP analogues (Fig. 6 H; similar data not shown for CD80, CD86, and MHCII).

DISCUSSION

In this paper, we have shown that endogenous PGD_2 acting at DP1 on BM-derived cells has an important feedback function in allergic inflammation and that administration of a selective DP1 agonist substantially suppresses the elicitation of inflammatory allergic reactions and bronchial hyperreactivity. Lung administration of DP1 agonist was able to suppress asthma equally well when given during Th2 priming in naive mice as during allergen challenge in already sensitized mice, suggesting that selective DP1 agonists might be used as a novel class of antiinflammatory drugs.

OVA-sensitized and challenged chimeric $DP1^{-/-}$ mice had strongly enhanced airway inflammation compared with chimeric WT mice. At first sight, our findings are in sharp contrast to data in constitutive $DP1^{-/-}$ mice that demonstrated less severe features of asthma (12). In our experiments, DP1 deficiency was restricted to BM-derived cells, possibly explaining the observed differences. It is known that structural cells of the airway wall such as epithelial cells, fibroblasts, and smooth muscle cells express the DP1 receptor (3, 12) and react to PGD_2 by producing Th2-selective chemokines such as CCL22, also known as MDC (17). In constitutive $DP1^{-/-}$ mice, the loss of this epithelial allergy-promoting mechanism is possibly dominant over the allergy-promoting effect of DP1 deficiency on hematopoietic cells, and consequently asthma severity would be reduced. Our data on enhanced Th2 responses in the absence of DP1 are also supported by studies in parasite infection. *Schistosoma* infection of the skin led to enhanced Th2 responses and lower levels of IFN-γ and IL-10 in $DP\text{-}1^{-/-}$ mice compared with WT mice (24).

It was previously shown that lipocalin-type PGD synthase transgenic mice had strongly enhanced airway inflammation, again contrary to our findings (5). The supraphysiological amount of PGD_2 produced in these transgenics could lead to high levels of downstream metabolites 9α-11β-PGF_2, 12-PGD_2, 15-deoxy-^{12,14}-PGD_2, and DK-PGD_2 that more selectively act on the chemotactic and proinflammatory DP2 receptor (CRTH2) on inflammatory cells (9) or could act by enhancing the epithelial release CCL22, attacking Th2 cells (17). It could also mean that in an integrated setting the proinflammatory effects of DP2 triggering override any protective effect of DP1 activation. In our model, we did not find enhancement of airway eosinophilia when PGD_2 or the DP2 agonist DK-PGD_2 was administered to the airways, in contrast to other reported work (14, 15, 17). The observed differences could be caused by subtle variations in the sensitization (use of alum adjuvant) and challenge protocol (number of OVA aerosols) used for inducing asthma.

When given by inhalation, a selective DP1 agonist suppressed all the cardinal features of asthma, including bronchial hyperreactivity. This suggests strongly that any proinflammatory effects on structural cells (epithelial cells, fibroblasts, and smooth muscle cells) (3) mediated by DP1 were not strong enough to overcome the protective effects of DP1 on hematopoietic cells. It is possible that the dose of agonist or its affinity for DP1 compared with PGD_2 reveals mainly the antiinflammatory effects of DP-1 ligation. Alternatively, BW245C was crossreacting with some other antiinflammatory receptor such as EP3 (11). Crossreactivity was, however, excluded by the finding that BW245C lost its antiinflammatory activity in $DP1^{-/-}$ mice. Only one effect of BW245C (reduction of CD40 expression on DCs) appeared to be DP1 independent.

Our findings in chimeric mice demonstrate that DP1 ligation on a BM-derived cell is important for the antiinflammatory effects of BW245C. The DP1, but not the DP2 (CRTH2) receptor, is expressed on mouse and human DCs (24, 25) and ligation of DP1 alters the migration of lung DCs (22). In view of the essential role of DCs in orchestrating lung immunity, we hypothesized that DP-1 agonism might be antiinflammatory by altering DC function (22). Supporting our hypothesis, in BW245C-treated animals or cells exposed in vitro to BW245C, DCs expressed less CD86, CD40, and MHCII, known to be essential for inducing naive and memory effector responses in the lungs (21, 26). We studied the dependency of this inhibition on DP1 and found that the effects of BW245C on CD86 and MHC II were DP1 dependent, whereas those on CD40 expression were DP1 independent both in vivo and in vitro. We have no explanation for these findings at present, but this suggests that some effects of BW245C on DCs could be through another receptor or through a metabolite using another receptor.

Further supporting an effect of DP1 ligation on DC function in the lung, we demonstrated that BW245C suppressed priming of Th2 responses via the airways in a model in which antigen presentation was restricted to endogenous mDCs (20). This was most likely a direct effect of the compound on DCs and not an indirect effect on epithelial cells as it could be mimicked by ex vivo BW245C treatment of BMDCs before adoptive transfer to the airways. Such adoptive transfer leads to stable Th2 priming and to asthmatic features in the lungs of recipients (19).

Reducing the migration and/or maturation of DCs is one likely explanation by which asthma features were suppressed, as the induction and maintenance of allergic lung inflammation depends on adequate function of mature lung DCs (18, 21). In all our in vivo (Fig. 1 D and Fig. 5 B) and in vitro (Fig. 6 B) experiments in which DCs were exposed to the DP1 agonist BW245C, we noticed a reduction or maintenance of Th2 cytokines in the induced T cell response, whereas the immunoregulatory cytokine IL-10 was increased. The most likely source of this enhanced IL- 10 was $CD4^+$ T cells (Fig. 1 F) as we could not detect any increase in intracellular IL-10 in BW245C-exposed DCs (Fig. 3 D). The low level proliferation and high level production of IL-10 in OVA-specific T cells stimulated by BW245C-treated OVA-DCs in vitro is reminiscent of adaptive T reg cells. Furthermore, OVA-specific $CD4^+$ T cells put in culture with BW245C-treated DCs strongly up-regulated Foxp3, a transcription factor found at high levels in natural and subsets of adaptive T reg cells. These $Foxp3^+$ $CD4^+CD25^+$ T reg cells,

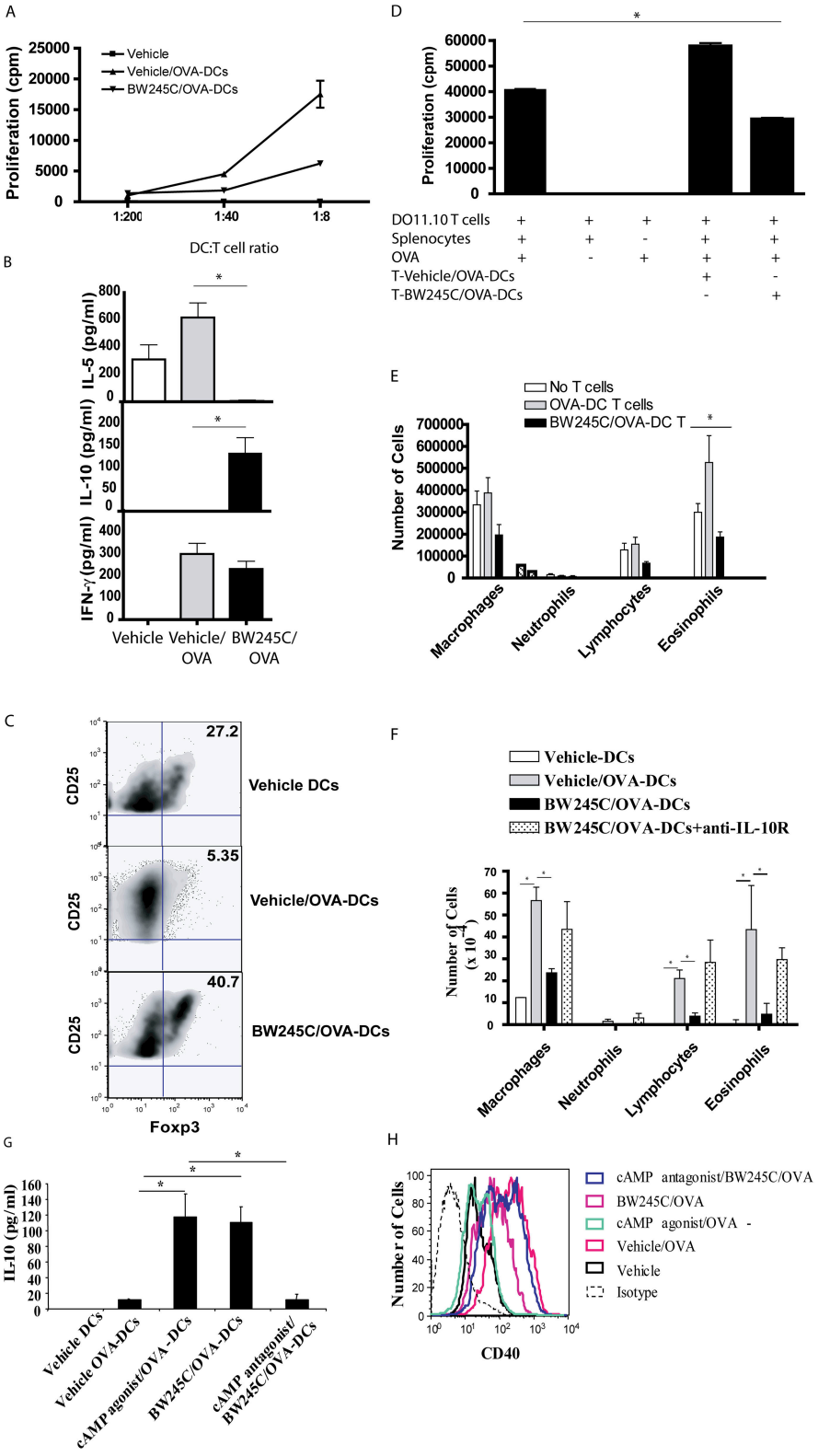

Figure 6. BW245C treatment of DCs induces T reg cells. Vehicle/OVA-DCs, BW245C/OVA-DCs, and unpulsed DCs (Vehicle DCs) were co-cultured for 4 d with naive OVA-specific $CD4^+$ T cells. (A) Cell proliferation after a 16-h pulse. (B) Cytokines in the supernatant. (C) Foxp3 expression. (D) Regulatory T cell assay in vitro. Proliferation of OVA-specific T cells after a 2-d co-culture with syngeneic splenocytes and OVA peptide in the presence of T cells previously stimulated with vehicle/OVA-DCs (T-Vehicle/OVA-DCs) or BW245C/OVA-DCs (T-BW-DCs). (E) Regulatory T cell assay in vivo. On day 0, mice received an i.t. injection of vehicle/OVA-DCs, BW245C/OVA-DCs, or unpulsed DCs (Vehicle DCs). From days 10–13, mice

known to suppress inflammation via IL-10 in vivo, were also increased in vivo after BW245C treatment (Fig. 1 F) and in mice receiving BW245C-treated DCs (Fig. 5) in a DP1-dependent manner. We do not know at present whether the increase in Foxp3$^+$ CD4$^+$CD25$^+$ cells is the result of expansion or recruitment of naturally occurring T reg cells or whether they are induced de novo from naive antigen-specific T cells in the periphery (27).

The main discriminating feature of induced and natural T reg cells is their capacity to reduce T cell proliferation upon co-culture with naive T cells or inflammation in vivo. Several data in our paper suggest that functional T reg cells were indeed induced. First, OVA-specific T cells that were initially stimulated by DP1-treated OVA-DCs partially inhibited the proliferation of fresh OVA-specific T cells. Second, T cells obtained from BW245C/OVA-DC–immunized mice and transferred to OVA-alum–sensitized mice partially inhibited OVA aerosol-induced eosinophilic airway inflammation in vivo, a classic example of T reg cell activity (23, 28, 29). Third, and most importantly, the immunomodulatory effect of BW245C on DCs in vivo was dependent on bioactive IL-10 from the host, as shown by IL-10R blocking experiments or transfer into IL-10–deficient recipients in vivo. By analogy, Kearley recently demonstrated that OVA-specific CD4$^+$CD25$^+$ T reg cells suppressed airway inflammation through induction of host IL-10 production (29). In IL-10–deficient mice, BW245C-treated DCs even induced stronger Th2 immunity and airway inflammation, best explained by the previous observation that PGD_2 treatment of splenic DCs in vitro suppresses IL-12p70 (30). It seems therefore that DP1 ligation of DCs endows these cells with a potential to induce both Th2 and T reg cell responses, but in vivo the production of IL-10 is able to suppress the effector functions of Th2 cells.

Exactly how T reg cells are induced by DP1 ligation of DCs is likely a complex phenomenon but could be related to the capacity of DP1 agonists to raise the levels of intracellular cAMP in DCs (30). Several compounds that alter DC function (such as histamine, β-adenergic agonists, or adenosine) do so by raising intracellular cAMP, and this might explain some of the antiinflammatory effects described with these compounds (31, 32). In support for this pathway, the IL-10–enhancing effects of BW245C-treated DCs on T cells were abolished when the downstream cAMP-dependent PKA was blocked using the inhibitor Rp8-Br-cAMP. Conversely, DCs treated with the cell-permeable cAMP analogue 8-bromium-cAMP also induced IL-10 production in responding T cells. Additionally, our preliminary experiments show a rise in nuclear-associated CREB- 1, a transcription factor binding to the consensus cAMP response element in nuclear extracts of BW245C-treated DCs. BW245C and 8-Br-cAMP (Fig. 6 H) treatment inhibits the LPS-driven maturation of DCs in vitro, and this process required PKA. Others have shown that immature DCs can induce a state of peripheral tolerance by induction of T reg cells (23, 27, 33, 34).

Our findings in DP1$^{-/-}$ chimeric mice, OVA-DP1$^{-/-}$ DC-immunized mice, and in vitro DP1$^{-/-}$ BM cultures suggest an endogenous source of PGD_2 that tonically inhibits airway inflammation by suppressing the maturation of DCs. In vivo, a predominant source of PGD_2 is from IgE-triggered mast cells that is mainly known to cause edema and cellular recruitment by directly acting on endothelial and inflammatory cells. It is tempting to speculate that PGD_2 down-regulates DC activation in an attempt to keep inflammation in check. Other predominant sources of PGD_2 are macrophages and DCs (35). Alveolar macrophages suppress the function of lung DCs by maintaining them in an immature state through secretion of PGs, but it has not been studied exactly which COX-dependent prostanoid (PGD_2 or PGE_2) is involved (36, 37). The availability of PGD2 synthase-deficient mice and use of reconstitution experiments in mast cell or macrophage-deficient mice will in the future solve these outstanding questions.

Finding novel ways to treat allergic inflammation is the goal of many laboratories in the world. Selective DP1 antagonists have been proposed as novel antiasthmatic drugs with the idea of inhibiting the proinflammatory effects of PGD_2. Our data caution against this strategy as eventually these drugs might potentiate allergic sensitization and inflammation. We favor the idea of finding selective DP1 agonists that exploit the full antiinflammatory effect on airway DCs.

MATERIALS AND METHODS

Mice. IL-10$^{-/-}$, C57BL/6, and BALB/c mice were from The Jackson Laboratory. DP1$^{-/-}$ mice (C57BL/6) were previously described (12). DO11.10 TCR transgenic mice were bred in house. To prepare BM chimeras, C57BL/6 mice were irradiated. All experiments were performed according to institutional ethical guidelines.

Preparation of BM chimeric mice. C57BL/6 mice were γ-irradiated twice with 5 Gy and were reconstituted with 2×10^6 BM cells from DP1-deficient or WT control mice (CD45.2). At 6–7 wk after reconstitution, chimeric mice were used for experiments.

OVA-alum model of asthma. Mice were sensitized to OVA (Worthington Biochemical) via the i.p. injection of OVA-alum on days 0 and 7, and

were exposed to OVA aerosols. On day 14, CD4$^+$ T cells were purified from MLNs and injected into recipient mice sensitized with OVA-alum 7 and 14 d earlier. 3 d after the CD4$^+$ T cell transfer, mice were exposed to three OVA aerosols. BAL fluid was analyzed by flow cytometry. (F) The inhibitory effect of BW245C treatment of DCs is dependent on IL-10. On day 0, mice were injected i.t. with unpulsed DCs (Vehicle DCs), vehicle/OVA-DCs, or BW245C/OVA-DCs. Some mice were also injected i.p. with blocking anti–IL-10R antibodies 1 d before aerosol exposure. BAL fluid was analyzed by flow cytometry. Data are mean value ± SEM. *, $P < 0.05$. (G) Effects of BW245C on DCs depend on cAMP-dependent PKA. DCs were pulsed with OVA in the presence of vehicle, the PKA inhibitor Rp8-Br-cAMP, or the cell-permeable cAMP analogue 8-Br-cAMP, subsequently washed and put into culture with naive DO11.10 T cells. IL-10 production was measured 4 d later in supernatants. (H) Effects of BW245C in suppressing DC maturation (as indicated by CD40 up-regulation) depend on PKA and are mimicked by 8-Br-cAMP.

were challenged with OVA aerosols on days 17–19, delivered from a jet nebulizer delivering 1% OVA in PBS for 30 min. 30 min before each OVA exposure, mice were anesthetized using avertin and received an intratracheal (i.t.) injection of vehicle, or of 100 μM of PGD2, DK-PGD2, or BW245C (Cayman Biocemical Corp.). 24 h after the last OVA exposure, BAL was performed and cells were stained with anti-CCR3 PE (R & D Systems), anti–I-Ad/I-Ed FITC, anti-CD3, and anti-B220 cy-chrome and anti-CD11c APC (all from BD Biosciences) and analyzed by flow cytometry (38). In brief, eosinophils were characterized as CCR3+ SSChi cells, neutrophils as CCR3− SSChi cells, lymphocytes as CD19+/CD3+ FSClo cells, and macrophages as large autofluorescent cells. After BAL was performed, lungs were resected, fixed, and embedded, and 5-μm sections were performed and stained with May Grunwald Giemsa.

In some experiments, lungs were collected and digested with collagenase III and DNase I (39). Single cell suspensions were stained with FITC anti–I-Ad/I-Ed, PE-labeled anti-CD40, anti-CD80, anti-CD86, and APC-labeled anti-CD11c antibodies (all from BD Biosciences).

To measure cytokine levels, MLN cells were plated in round bottom 96-well plates (10^6 cells/ml) and restimulated with OVA (10 μg/ml) for 4 d. Production of IL-5, IL-10, and IFN-γ was assayed on supernatants by ELISA (BD Biosciences).

For intracellular cytokine staining, MLN cells were restimulated in vitro for 5 h with PMA, ionomycin, and brefeldin A (all from Sigma-Aldrich). Cells were washed and stained with FITC-labeled CD25 and APC-labeled anti-CD4 (BD Biosciences). After washing, cells were permeabilized for 20 min and stained with PE-labeled anti–IL-10 antibodies (BD Biosciences). For demonstrating T reg cells, cells were stained with PE-labeled anti-Foxp3 antibodies (eBioscience).

Th2 sensitization induced by endogenous mDCs. Mice were depleted of pDCs by three i.p. injections of anti–Gr-1 antibody (250 μg/mouse) as described (20). On day 0, 800 μg of OVA was injected i.t., admixed with 100 μM of BW245C or vehicle. After a wash out period of 10 d, mice received three OVA aerosols of 30 min on three consecutive days, and mice were killed 24 h later.

Th2 sensitization induced by i.t. injection of BM-derived mDCs. Bone marrow cells were grown in medium containing recombinant murine GM-CSF (200 IU/ml) (19). At day 9 of culture, cells were pulsed overnight with 100 μg/ml OVA in the presence of 10 μM BW245C or DMSO vehicle. Nonadherent DCs were collected, washed to remove free OVA or BW245C, 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% vol/vol in PBS), and 10^6 vehicle DCs, vehicle/OVA-DCs, or BW245C/OVA-DCs were instilled through the opening vocal cords as described (19). On days 10–12, mice were exposed to a 30-min OVA aerosol and killed 24 h later.

In some experiments, vehicle/OVA-DC and BW245C/OVA-DC–immunized mice were injected i.p with 500 μg of blocking anti–IL-10R antibodies (aIL-10R) (BD Biosciences) 1 d before aerosol challenge. Alternatively, BMDCs were derived from WT C57BL/6 mice, treated or not with BW245C, and injected i.t. into WT or IL-$10^{-/-}$ recipient mice.

Determination of BHR. 24 h 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 (MCH) using ultrasonic nebulizers. PenH values were measured 3 min after each metacholine aerosol using a whole body plethysmograph (Buxco, Electronics). The average PenH values were expressed for each MCH concentration as the percentage increase over baseline PenH values.

For invasive measurement of dynamic resistance and compliance, mice were anesthetized with urethane, paralyzed using D-tubocurarine, tracheotomized, and intubated with an 18-G catheter, followed by mechanical ventilation with a Flexivent apparatus (SCIREQ). Respiratory frequency was set at 120 breaths/min with a tidal volume of 0.2 ml, and a positive-end expiratory pressure of 2 ml H_2O was applied. Increasing concentrations of metacholine (0–600 $\mu g/kg^{-1}$) were administered via the jugular vein. Dynamic resistance and compliance were recorded after a standardized inhalation maneuver given every 10 s for 2 min. Baseline resistance was restored before administering the subsequent doses of MCH.

Naive T cell activation and differentiation in vitro. 2×10^5 OVA-specific TCR transgenic $CD4^+$ T cells obtained from DO11.10 mice were cultured in vitro in round bottom 96-well plates for 4 d with increasing numbers of BALB/c OVA-DCs pretreated with vehicle or with BW245C. In some experiments, DCs were also pretreated with the cAMP analogue 8-Br-cAMP or with the PKA inhibitor Rp8-Br-cAMP. Cell proliferation of naive T cells was assessed by ^{3}H-thymidine (1 μCi/well) uptake in a 16-h pulse. To assess Th cell differentiation, supernatants were harvested and analyzed by ELISA.

Induction of regulatory T cells. DCs pulsed with OVA, treated with vehicle or with BW245C, were cultured with naive DO11.10 $CD4^+$ T cells for 3 d at a 1:10 ratio (20). T cells were harvested and cultured at 5×10^5 cells per well without OVA in the presence of 1 ng/ml mouse IL-2 (R & D Systems) for an additional 7 d. Regulatory activity was assayed by culturing freshly purified DO11.10 $CD4^+$ T cells (10^5 per well) with irradiated BALB/c splenocytes (10^5 per well) in 96-well plates, with 10 μg/ml $OVA_{323-339}$ peptide, in the presence or absence of DC-stimulated T cells (10^5 per well). Cell proliferation was assessed after 48 h by ^{3}H-thymidine (1 μCi/well) uptake in a 16-h pulse.

To address induction of T reg cells in vivo, mice received BW245C-treated DCs or OVA-pulsed DCs and were challenged with OVA aerosol from days 10–12. On day 13, MLNs were collected and $CD4^+$ T cells were purified (purity >95% using magnetic bead purification; Miltenyi Biotec). 2×10^6 $CD4^+$ cells were injected i.v. into mice that were sensitized twice by injection of OVA-alum. 3 d after injection of $CD4^+$ T cells mice were exposed to three OVA aerosols.

Statistical analysis. For all experiments, the difference between was calculated using the Mann Whitney U test for unpaired data. Differences were considered significant if $P < 0.05$. Groups consisted of at least eight mice per group. Experiments shown are representative of three experiments.

Online supplemental material. Fig. S1 shows numbers of cells in the BAL fluid of WT or IL-$10^{-/-}$ mice injected i.t. with vehicle/OVA-DCs, BW245C/OVA-DCs, or unpulsed DCs. Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20061196.

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