

Activation of the Prostaglandin D₂ Receptor DP2/CRTH2 Increases Allergic Inflammation in Mouse¹

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Allergic pathologies are often associated with IgE production, mast cell activation, and eosinophilia. PGD₂ is the major eicosanoid, among several inflammatory mediators, released by mast cells. PGD₂ binds to two membrane receptors, D prostanoid receptor (DP)1 and DP2, endowed with antagonistic properties. In humans, DP2 is preferentially expressed on type 2 lymphocytes, eosinophils, and basophils and mediates chemotaxis *in vitro*. Although not yet supported by *in vivo* studies, DP2 is thought to be important in the promotion of Th2-related inflammation. Herein, we demonstrate that mouse eosinophils express both DP1 and DP2 and that PGD₂ exerts *in vitro* chemotactic effects on eosinophils through DP2 activation. Furthermore, 13,14-dihydro-15-keto-PGD₂, a specific DP2 agonist not only increases eosinophil recruitment at inflammatory sites but also the pathology in two *in vivo* models of allergic inflammation: atopic dermatitis and allergic asthma. By contrast, DP1 activation tends to ameliorate the pathology in asthma. Taken together, these results support the hypothesis that DP2 might play a critical role in allergic diseases and underline the interest of DP2 antagonists in human therapy. *The Journal of Immunology*, 2005, 174: 3703–3708.

Allergic reactions such as asthma or atopic dermatitis (AD)³ are inappropriate inflammatory responses that develop against environmental allergens. These reactions are usually associated with a Th2-polarized immune response, including Ag-specific IgE production and local recruitment of inflammatory cells such as eosinophils and mast cells.

Mast cells indirectly participate in asthmatic reactions (1). IgE-dependent mast cell activation exacerbates development of airway hyperresponsiveness (AHR) (2). Presence of mast cells also potentiates AD (3). Upon activation by IgE and multivalent Ag, mast cells release several inflammatory mediators such as histamine, proteases, cytokines, and eicosanoids, including leukotrienes and PGs. Among the later, PGD₂ is the most abundantly produced. During acute asthmatic episodes, PGD₂ is released by mast cells into the lungs (4) and causes bronchoconstriction (5). PGD₂, as well as histamine, directly activates eosinophils (6), promotes their recruitment (7), and affects other key parameters of lung inflammation, in particular vascular permeability (1). The role of PGD₂ in eosinophilia and lung allergic response has been recently demonstrated using transgenic (Tg) mice that overexpress PGD₂ synthase (8). Furthermore, PGD₂ nebulization before aerosol Ag challenge enhances Th2-type inflammatory re-

sponses, including eosinophilia, and leads to the development of AHR by increasing bronchial expression of macrophage-derived chemokines (9).

PGD₂ directly acts through the D prostanoid receptor (DP)1 (G_{αs} coupled) and DP2 (also known as chemoattractant receptor-homologous molecule expressed on Th2 cells or CRTH2) (G_{αi} coupled), two membrane-bound receptors that exert broadly antagonistic effects. Within the immune system, DP1 activation affects the maturation process and the migratory ability of human and mouse dendritic cells (DC), a key cell population involved in the initiation and the regulation of the immune response (10–12). On the other hand, DP2 was identified in humans on type 2 polarized lymphocytes (Th2 and Tc2) (13), basophils, eosinophils (14), and monocytes (12). Previous studies have revealed that DP2 mediates eosinophil chemotaxis induced by mast cell products (14) and was later identified as a PGD₂ receptor (15). DP2 activation thus accounts for the PGD₂-induced eosinophil chemotaxis and degranulation, whereas DP1 activation delays their apoptosis onset (16). Finally, the number of DP2⁺ cutaneous lymphocyte Ag⁺ lymphocytes is increased in patients with AD (17), whereas DP1 activation decreased inflammation in a murine model of AD (18).

Most of the studies on DP2 have been undertaken on human eosinophils *in vitro*. Data on DP2 in mice are scarce. Mouse DP2 shares a 77% homology with its human counterpart (19) and is activated by PGD₂, 13,14-dihydro-15-keto-PGD₂ (DK-PGD₂), 15-deoxy-Δ^{12,14}-PGJ₂, and indomethacin (20). However, DP2 is not preferentially expressed on Th2-type cells, as it is in humans, and is found on both Th1 and Th2 clones (19 and our unpublished observations). DP2 function might thus be different in mice and humans. Since no data are available so far on DP2 functions *in vivo*, the use of animal models is required to delineate its role in both physiology and pathology. We thus investigated DP2 involvement in allergic pathologies, in particular in eosinophilia, using two mouse models of allergic inflammation, faithfully mimicking the corresponding human diseases: asthma and AD. In these models, we show that DP2 activation exacerbates pathology.

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³ Abbreviations used in this paper: AD, atopic dermatitis; AHR, airway hyperresponsiveness; Tg, transgenic; DP, D prostanoid receptor; DC, dendritic cell; DK-PGD₂, 13,14-dihydro-15-keto-PGD₂; BAL, bronchoalveolar lavage; MGG, May-Grunwald-Giemsa.

Materials and Methods

Mice

Female BALB/cJ mice (6–8 wk old) were purchased from Charles River and kept at the Institut Pasteur de Lille under specific pathogen-free conditions. Tg mice expressing IL-5 under the control of human CD2 promoter (21) were used for eosinophil purification. Experiments were performed according to local ethical guidelines.

Cell preparation

Eosinophils were purified from IL-5 Tg mouse spleen by negative selection, as described (22), using anti-CD8 α , anti-CD90, and anti-B220 Abs (BD Pharmingen). Purity was >90%.

RNA isolation and RT-PCR

Total RNA was isolated using RNAsplu (Qiogene). Reverse transcription was performed with 1 μ g of RNA using SuperScript RT. Mouse DP1- and DP2-specific fragments and β -actin were amplified using the following primers and conditions. DP1, 5'-GAAGTTCGTGCAGTACTGTCCAG-3' (sense); 5'-TCCACTATGGAAATCACAGAC-3' (antisense). DP2, 5'-CATGTGCTACTACAACCTTGC-3' (sense); 5'-GCAGACTGAAGATGTGGTAGG-3' (antisense). β -Actin, 5'-TCACCGAGGCCCCCTGAAC-3' (sense); 5'-GCACGCACTGTAATTCCTC-3' (antisense). Annealing temperature was 54, 55, and 60°C for DP1, DP2, and β -actin, respectively. Amplification was performed for 40 cycles for DP1 and DP2 and for 25 cycles for β -actin. Amplicon size was 435, 262, and 324 bp for DP1, DP2, and β -actin, respectively. To exclude potential amplification from contaminating genomic DNA, control experiments, where the reverse transcription step was omitted, were performed. Gel loadings of amplified products were normalized according to the signal from β -actin. KmDP53 and KmB20 cells (K562 cells, respectively, transfected by mouse DP1 and DP2 cDNA) were kindly provided by Prof. K. Sugamura (Tohoku University, Japan).

Chemotaxis assay

Eosinophil chemotaxis assay was performed in Boyden chambers as described (23). BW245C and DK-PGD₂ (Cayman Chemical) as well as CCL11 (Preprotech) were used as chemoattractants. Except for CCL11, stock solutions (10⁻² M) were prepared in DMSO (Sigma-Aldrich). Cells that had migrated and adhered to filter were counted from four fields for each experimental condition.

Ag-induced AHR

Mice were sensitized by i.p. injection with 50 μ g of OVA in 100 μ l of alum (Imject; Pierce) or received alum only and were challenged for 20 min, on days 14, 16, 18, 20, and 22 by aerosol nebulization with OVA (1% in PBS) using an ultrasonic nebulizer (Systam). Groups of unsensitized or sensitized animals were additionally nebulized with 50 μ M DK-PGD₂ or BW245C (stock 10⁻² M in ethanol) in PBS for 20 min immediately before and during each OVA challenge (23), whereas the corresponding untreated groups were nebulized with an equivalent amount of ethanol. Serum was collected on day 23, and AHR to increasing concentrations of methacholine was measured by whole body plethysmography (Emka) on day 24. Results were expressed as Penh values (24). Lungs were used either for bronchoalveolar lavages (BAL) or for histological analyses and determination of cytokine content in protein extracts. Cytokine content was determined in lung protein extracts (250 μ l per right lung) prepared as described (23) using specific ELISA (R&D Systems) for IL-5. BAL were analyzed on cytosin preparations following RAL 555 staining.

Atopic dermatitis

AD was induced by epicutaneous sensitization with OVA as described (25). Two paper disc inserts of a Finn Chamber (Promedica) were applied on abdominal skin 24 h after shaving after soaking with 25 μ l of OVA solution (2 mg/ml in PBS) or with PBS. Patches were secured to the skin with a bioocclusive dressing (Visulin; Hartmann), itself protected with an elastic bandage (Optiplaste; Smith & Nephew). Patches were left on for three 1-wk periods (with patch renewal at mid-week) with a 2-wk interval between application. Immediately before patch application, abdomens were topically treated with 25 μ l of 50 μ M DK-PGD₂ (stock 10⁻² M in DMSO) in acetone/olive oil (4:1 v/v), whereas the corresponding untreated mice were receiving an equivalent volume of DMSO. Blood was collected on day 49, at the time of last patch removal, and animals were sacrificed on the next day by cervical dislocation. Skin samples were collected for histological analyses.

Histology

Samples were fixed in ImmunoHistoFix (Intertiles) for 7 days at 4°C, then included in ImmunoHistoWax (Intertiles) after ethanol dehydration. Serial 5- μ m transversal sections were prepared, dewaxed in acetone, and stained with May-Grunwald-Giemsa (MGG) for general histology and eosinophil counts and with acidic toluidine blue for mast cell counts. For each skin section, 10 random fields were examined at 1000-fold magnification. The various cell types in dermis were enumerated using an eyepiece equipped with a calibrated grid. Results were expressed as cell number per square millimeter. Epidermal thickness was determined at 200-fold magnification with an ocular micrometer. The average of 10 measures was calculated for each sample. For lung sections, the number of eosinophils was determined by counting the total number of eosinophil present in a whole lung section and by measuring the total surface of the lung section (three non-serial sections were used for each sample). Results were expressed as cell number per square millimeter.

Measurement of Ig concentrations

Serum anti-OVA IgG₁ were measured by ELISA, using OVA-coated plates and HRP-conjugated anti-mouse IgG₁ (Southern Biotechnologies) (23). Serum anti-OVA IgE was measured by ELISA using anti-IgE (BD Pharmingen) as previously described (23). Biotinylated Ab and biotinylated OVA and HRP-conjugated streptavidin (Amersham Biosciences) were used for detection. Serial dilutions (2-fold) were prepared (starting dilution 1/25 for IgE and 1/5000 for IgG₁ titrations). Ab titers were calculated as the dilution corresponding to twice the mean absorbance value obtained for non-sensitized mouse sera. Total IgE concentrations were measured by ELISA using two monoclonal anti-IgE Abs (BD Pharmingen) as previously described (26).

Statistical analyses

Statistical significance was determined with the Statview software using Student's *t* test except for chemotaxis and plethysmography data for which ANOVA for repeated measures was used. Results were expressed as mean \pm SEM; *p* < 0.05 was considered significant.

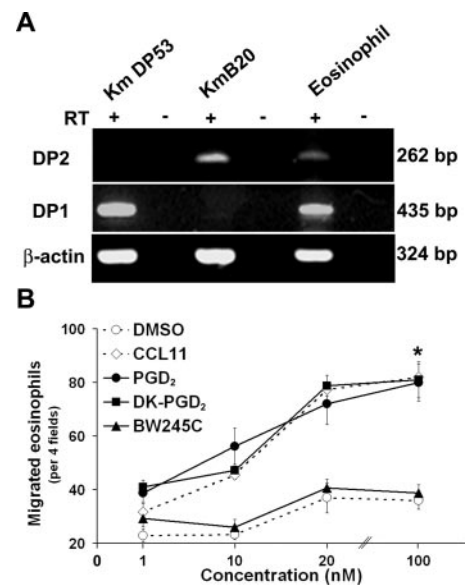


FIGURE 1. Expression and function of DP1 and DP2 on mouse eosinophils. *A*, RT-PCR amplification of DP2, DP1, and β -actin mRNA from mouse eosinophils and transfected K562 cells expressing DP1 (KmDP53) or DP2 (KmB20). PCR reactions were performed following (+) or in the absence of (-) a RT step. *B*, Dose-dependent chemotaxis of mouse eosinophils toward PGD₂, DK-PGD₂, BW245C, and CCL11 as assessed in Boyden chamber. Each data point represents quadruplicate experiments. *, *p* < 0.05 (compared with vehicle).

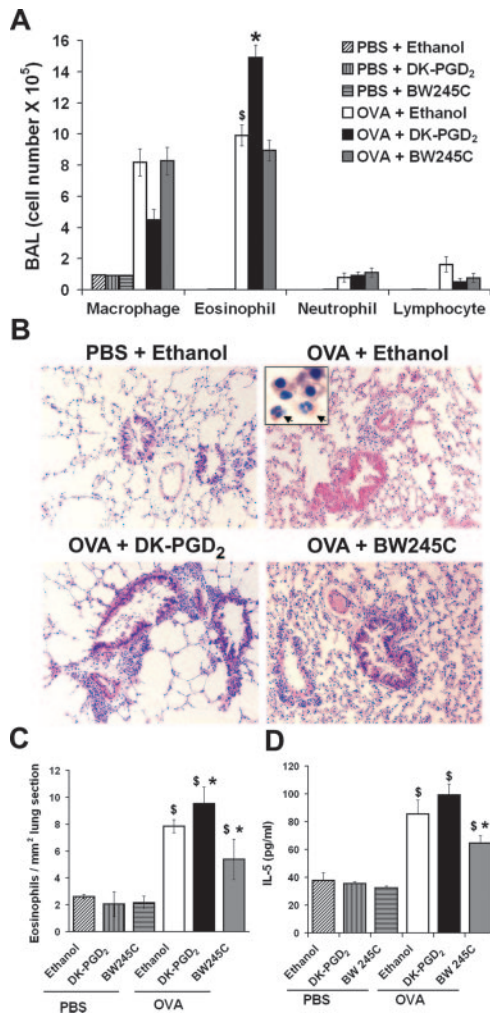


FIGURE 2. DK-PGD₂ exacerbates lung inflammation in a mouse model of asthma. Mice were sensitized by i.p. injection of OVA in alum and challenged by repeated OVA nebulizations together with DK-PGD₂ (50 μ M), BW245C (50 μ M), or vehicle. Unsensitized control mice received alum only and were challenged with OVA and treated with DK-PGD₂, BW245C, or vehicle as for sensitized mice. Mice were sacrificed 48 h after the last nebulization. *A*, Total number of macrophages, eosinophils, lymphocytes, and neutrophils in BAL at the time of sacrifice ($n = 3-8$ animals per group). *B*, MGG staining of lung sections from sensitized and challenged mice treated with ethanol, DK-PGD₂, or BW245C or from PBS-sensitized but challenged animals treated with ethanol (original magnification $\times 100$). *Inset*, arrows indicate eosinophils (original magnification $\times 630$). *C*, Number of eosinophils per surface of lung section ($n = 3-5$ animals per group). *D*, IL-5 concentrations in lung protein extracts ($n = 3-5$ animals per group). *, $p < 0.05$ vs OVA-sensitized and ethanol-treated mice. \$, $p < 0.05$ vs PBS-sensitized and ethanol-treated mice.

Results

DP1 and DP2 transcripts are expressed in mouse eosinophils

Since DP2 is expressed on human eosinophils and basophils, besides Th2 and Tc2 lymphocytes (15), we first intended to determine whether this was also the case for mouse eosinophils. We thus analyzed, by RT-PCR, DP1 and DP2 steady-state mRNA levels in purified eosinophils, isolated from IL-5 Tg mice. Cells transfected with mouse DP1 (KmDP53) and DP2 (KmB20) cDNA were used as positive controls. Expression of both receptors was detected in eosinophils (Fig. 1A). Eosinophils appear to express greater amounts of DP1 mRNA compared with DP2 mRNA. Thus, although the lack of specific Abs did not allow us to confirm these

data at the protein levels, PGD₂ might act on eosinophils through activation of either DP1 or DP2.

DP2 activation mediates mouse eosinophil migration in vitro

Since PGD₂ is involved in cell chemotaxis (12, 15, 27, 28), we next examined the effects of DP1 and DP2 agonists on eosinophil migration in vitro. PGD₂, BW245C (a specific DP1 agonist) or DK-PGD₂ (a specific DP2 agonist) were tested for their ability to attract eosinophils. As represented in Fig. 1B, eosinophils were not only attracted by the chemokine CCL11 (used as a positive control) but also by PGD₂ and DK-PGD₂ in a dose-dependent manner. On the other hand, BW245C was without effect. Therefore, DP2 is functional in mouse eosinophils and promotes, as is the case in humans, eosinophil chemotaxis in vitro.

DP2 activation increases inflammation and eosinophilia in an asthma model

Having established that DP2 exerts a chemotactic activity on mouse eosinophils in vitro, we next intended to study its role in two in vivo models of allergic reactions associated to eosinophilia, namely asthma and AD. Due to the apparently antagonistic role of DP1 and DP2 (for instance on cell migration), we also studied the effect of DP1 activation in experimental asthma.

BALB/c mice were sensitized with OVA and treated by nebulization with DK-PGD₂ or BW245C at the time of each Ag challenge and were compared with vehicle (DMSO)-treated animals. OVA sensitization and challenge induced eosinophilia in BAL and lung tissue, mainly in perivascular areas, compared with control animals (Fig. 2, A-C). In sensitized and challenged animals, treatment with DK-PGD₂ further increased both BAL and tissue eosinophilia, whereas treatment with BW245C did not affect BAL eosinophilia but slightly decreased tissue inflammation and eosinophilia (Fig. 2, A-C). Interestingly, among the cytokines tested (IL-4, -5, -6, -10, -13, and IFN- γ), the decreased eosinophilia observed in BW245C-treated animals was accompanied by a significant decrease in lung IL-5 content (Fig. 2D), whereas the increase in lung IL-5 content induced by DK-PGD₂ was not statistically significant.

Along the same lines, lung function was also affected by OVA sensitization and challenge. Indeed, AHR to increasing doses of methacholine, monitored by whole body plethysmography on conscious unrestrained animals 48 h after the last Ag challenge, was increased compared with PBS-sensitized animals, as evidenced by higher Penh value, a dimensionless parameter reflecting the intensity of airway response (Fig. 3). Interestingly, DK-PGD₂-treated animals displayed increased AHR compared with DMSO-treated,

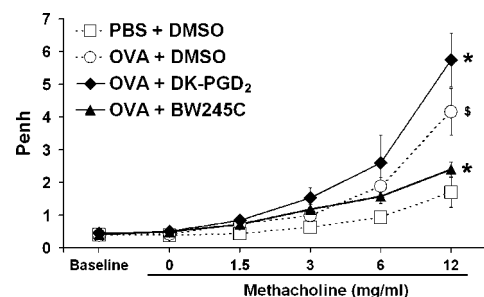


FIGURE 3. DK-PGD₂ exacerbates lung inflammation AHR in a mouse model of asthma. Mice were treated as described in Fig. 2 and AHR to increasing methacholine concentrations was measured by whole body plethysmography 48 h after the last nebulization. ($n = 6-13$ animals per group). *, $p < 0.05$ vs OVA-sensitized and ethanol-treated mice. \$, $p < 0.05$ vs PBS-sensitized and ethanol-treated mice.

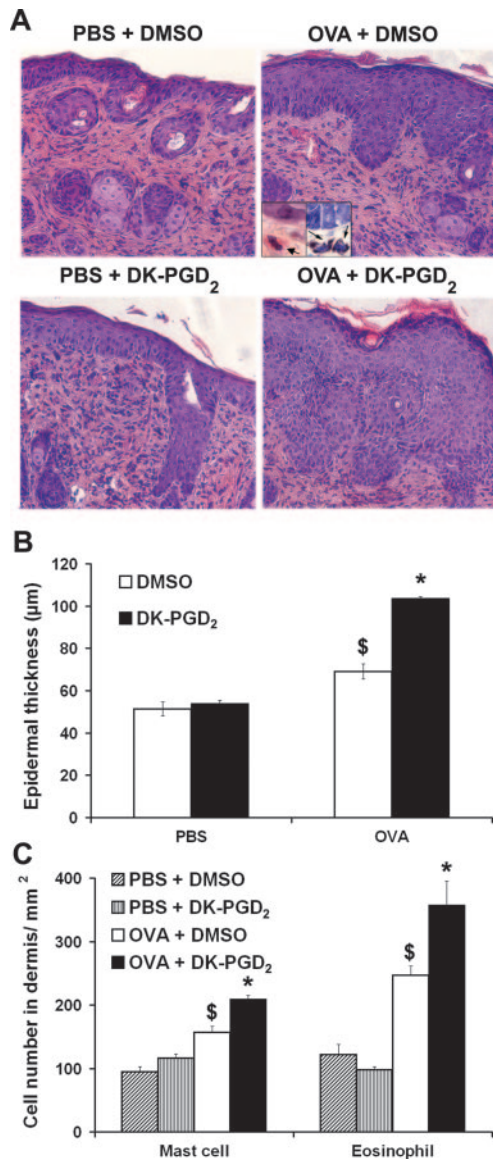


FIGURE 4. DK-PGD₂ exacerbates skin inflammation in a mouse model of AD. Mice were sensitized by three 1-wk periods of epicutaneous OVA or PBS application, each 2 wk apart, and topically treated with 50 µM DK-PGD₂ or vehicle at the time of each sensitization. Mice were sacrificed 24 h after the end of the last sensitization period. **A**, MGG staining of skin sections from OVA-sensitized or PBS-treated mice treated with DMSO or DK-PGD₂ (original magnification ×100). *Insets*, thick arrow indicates an eosinophil, thin arrows indicate mast cells (original magnification ×630). **B**, Epidermal thickness. **C**, Eosinophil and mast cell numbers in dermis at the site of sensitization and treatment. Enumeration was performed on MGG-stained sections for eosinophils and on acidic toluidine blue staining for mast cells. ($n = 6-13$ animals per group). *, $p < 0.05$ vs OVA-sensitized and DMSO-treated mice. \$, $p < 0.05$ vs PBS-sensitized and DMSO-treated mice.

but similarly sensitized and challenged, animals, whereas treatment with BW245C had the opposite effect and reduced AHR (Fig. 3). It is worth mentioning that, whatever the parameters analyzed, DK-PGD₂ and BW245C treatments were without significant effect in PBS-sensitized, OVA-challenged animals thus suggesting that they are only active in Ag-sensitized animals in which an inflammatory response takes place.

Finally, serum Ag-specific IgE and IgG₁ were measured following OVA sensitization and challenge. DK-PGD₂ or BW245C treat-

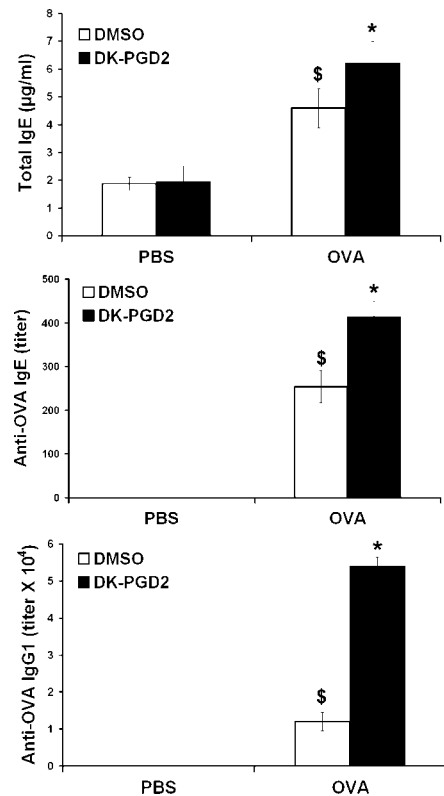


FIGURE 5. DK-PGD₂ exacerbates Th2 humoral response in a mouse model of AD. Mice were treated as described in Fig. 4. Blood was collected 24 h before sacrifice. Serum concentrations of total IgE, Ag-specific IgE, and IgG₁ were determined ($n = 6-13$ animals per group). *, $p < 0.05$ vs OVA-sensitized and DMSO-treated mice. \$, $p < 0.05$ vs PBS-sensitized and DMSO-treated mice.

ments did not significantly affect anti-OVA IgE concentrations. However, BW245C reduced anti-OVA IgG₁ titers (by 40%, not shown).

DP2 activation increases inflammation and eosinophilia in a model of AD

We next assessed the effect of DK-PGD₂ in a model mimicking human AD and based on Ag sensitization by repeated epicutaneous OVA applications in the absence of an adjuvant. This sensitization not only leads to the development of skin inflammation (Fig. 4A), characterized by epidermal thickening (Fig. 4B), hyperkeratosis, spongiosis, and dermal inflammatory infiltrates (Fig. 4C), but also to Ag-specific IgE and IgG₁ production. DK-PGD₂, topically delivered at the time of each sensitization, significantly exacerbated the pathology but had little if any effect in non-sensitized mice. Indeed, DK-PGD₂ treatment increased OVA-induced inflammation (Fig. 4A), epidermal thickening (Fig. 4B), and dermal infiltrates of mast cells and eosinophils (Fig. 4C), compared with untreated sensitized mice. Likewise, compared with untreated animals, DK-PGD₂ significantly increased the Th2-associated humoral response as reflected by higher levels of total and OVA-specific IgE and OVA-specific IgG₁ (Fig. 5).

Discussion

Several studies have shown that PGD₂ participates in the development of Th2-type inflammatory reactions, including allergic asthma (8, 9). In vivo, the contribution of DP1 in promoting or inhibiting lung eosinophilia and inflammation is controversial, whereas that of DP2 remains totally unexplored. In a similar way,

the role of DP2 activation in AD has never been investigated, whereas the protective role of DP1 in AD has recently been evidenced (18). Recent studies performed in vitro on human cells, inferred that DP2 might be an important promoter of Th2-related inflammatory reactions, particularly through its ability to mediate the recruitment of some inflammatory cells into peripheral sites and/or through its capacity to modulate their effector functions (15, 16, 29–31). However, although DP2 is a potential therapeutic target in humans to limit Th2-related inflammation, this assumption needs to be supported by in vivo studies. Herein, we show that DP2 is functional in mouse eosinophils and that it exerts promoting effects on eosinophilia and inflammation in both asthma and AD.

We have first demonstrated that mouse eosinophils express DP2 mRNA and provided evidences that DP2 activation induces their migration in vitro. This confirms data obtained with human cells (15, 16, 30, 31) but contrasts with a recent study claiming that DP2 is inactive in murine eosinophils, at least in terms of cell migration (32). This discrepancy might be explained by differences in the animals models (IL-5 Tg mice) used for eosinophil purification (cadmium-induced metallothionein-driven ubiquitous expression (33) vs CD2-driven T cell-specific expression in this study (21)) and/or in cell purification protocol (positive selection vs negative selection in our case). Nevertheless, mouse DP2 signal transduction pathways and biological effects appear similar to those involved in the workings of human DP2 (32). Indeed, ligand binding to the mouse DP2 induces Ca^{2+} mobilization and activation is sensitive to *Bordetella pertussis* toxin but not to cholera toxin, thus pointing toward receptor coupling to G_i proteins (32).

More importantly, we show for the first time that DP2 activation in vivo promotes eosinophilia and exacerbates pathology in two models of Th2-related inflammation: allergic asthma and AD.

The mechanisms accounting for DK-PGD₂ action in vivo remain unclear, although they likely involve a direct chemotactic effect on eosinophils. This direct effect is of particular relevance in asthma since it has recently been demonstrated, using two strains of eosinophil-deficient mice, that this cell type was crucial to the development of airway remodeling (34, 35) and, according to one report, to airway hyperreactivity (34). As various cell types express DP2 in mouse, DK-PGD₂ might also act on other cell populations such as T lymphocytes, as suggested by the slight increase in IL-5 in the lungs. Indeed, in humans, DP2 engagement appears to promote T cell activation and Th2-type cytokine release (36). However, such a finding has not yet been formally demonstrated in mice. Moreover, PGD₂ has recently been shown to activate airway epithelial cells to produce macrophage-derived chemokine, which in turn favors pulmonary eosinophilia and AHR (36). DP2 activation in airway epithelial cells might possibly account for this phenomenon. Finally, mast cells express DP2 mRNA (data not shown), and their number is increased by DK-PGD₂ in the AD model. It is possible that DP2 is active in cutaneous mast cells. Production of Ag-specific Abs is another key parameter of allergic pathologies that was increased by DK-PGD₂ treatment in the AD model but not in asthma. This probably reflects the fact that animals were treated over the entire duration of the experimental protocol in AD and only during the challenge phase in asthma. DP2 activation during the early phases of the immune response might indeed impact on DC functions and/or promote the recruitment of DC progenitors to peripheral sites, as recently suggested (12).

The role of DP1 in Th2-related inflammatory reactions remains controversial. DP1-deficient mice have been shown to display decreased eosinophilia in an asthma model (37) and a new DP1 antagonist has been shown to decrease eosinophilia in a guinea pig model of allergic rhinitis (38). By contrast, our present results

show that DP1 activation decreases eosinophilia and AHR in murine asthma. These apparently conflicting results on DP1 functions in the control of Th2-associated inflammation might be due to differences between animal species or mouse strain and/or between experimental approach to study DP1 (genetic inactivation in the whole animal vs airway targeting by aerosol treatment with agonist/antagonist). Finally, timing of DP1 activation might be of particular importance in the subsequent development of local immune-inflammatory response. Indeed BW245C treatment during the sensitization phase reduced the ability of Ag-loaded DCs to locally activate Ag-specific T cells in both AD (18) and asthma (H. Hammad, unpublished observations) models. Whether or not DP1 activation by BW245C during the challenge phase in the present asthma model alters DC functions still needs to be addressed. Moreover, DP1 activation might also inhibit the function of other cell types as demonstrated, at least in vitro, for eosinophils, whose CCL11-driven chemotaxis was also inhibited by BW245C (data not shown).

Taken together, our data reveal an important role of DP2 in promoting Th2-associated skin and lung inflammatory responses in mouse, whereas DP1 activation leads to the opposite outcomes and contributes to counter-regulate Th2 inflammation. This suggests that DP2 antagonists might be of therapeutic interest in diseases where eosinophilia has to be prevented. The consequences of DP2 activation by endogenously produced PGD₂ await further studies using DP2-deficient mice.

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Disclosures

The authors have no financial conflict of interest.

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