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Claudia Traidl-Hoffmann, Valentina Mariani, Hubertus Hochrein, Kathrin Karg, Hermann Wagner, Johannes Ring, Martin J. Mueller, Thilo Jakob, Heidrun Behrendt

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Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization

Claudia Traidl-Hoffmann,[1] Valentina Mariani,[1] Hubertus Hochrein,[2] Kathrin Karg,[4] Hermann Wagner,[2] Johannes Ring,[3] Martin J. Mueller,[4] Thilo Jakob,[1,3] and Heidrun Behrendt[1]

[1]ZAUM-Center for Allergy and Environment, Division of Environmental Dermatology and Allergy GSF/TUM, [2]Institute of Medical Microbiology, Immunology and Hygiene, and [3]Department of Dermatology and Allergy Biederstein, Technische, Universität München, 80802 Munich, Germany
[4]Julius-von-Sachs-Institute of Biosciences, Division of Pharmaceutical Biology, University of Würzburg, 97082 Würzburg, Germany

Pollen grains induce allergies in susceptible individuals by release of allergens upon contact with mucosal membranes of the upper respiratory tract. We recently demonstrated that pollen not only function as allergen carriers but also as rich sources of bioactive lipids that attract cells involved in allergic inflammation such as neutrophils and eosinophils. Here we demonstrate that soluble factors from birch (*Betula alba* L.) pollen activate human dendritic cells (DCs) as documented by phenotypical and functional maturation and altered cytokine production. *Betula alba* L. aqueous pollen extracts (*Bet.*-APE) selectively inhibited interleukin (IL)-12 p70 production of lipopolysaccharide (LPS)- or CD40L-activated DC, whereas IL-6, IL-10, and TNFα remained unchanged. Presence of *Bet.*-APE during DC activation resulted in DC with increased T helper type 2 (Th2) cell and reduced Th1 cell polarizing capacity. Chemical analysis of *Bet.*-APE revealed the presence of phytoprostanes (dinor isoprostanes) with prostaglandin E_1-, F_1-, A_1-, or B_1-ring systems of which only E_1-phytoprostanes dose dependently inhibited the LPS-induced IL-12 p70 release and augmented the Th2 cell polarizing capacity of DC. These results suggest that pollen-derived E_1-phytoprostanes not only resemble endogenous prostaglandin E_2 structurally but also functionally in that they act as regulators that modulate human DC function in a fashion that favors Th2 cell polarization.

CORRESPONDENCE
Thilo Jakob:
thilo.jakob@gsf.de

Abbreviations used: *Bet.*-APE, *Betula alba* L. aqueous pollen extracts; LAL, *Limulus* amebocyte lysate; NCI GC-MS, negative chemical ionization gas chromatography-mass spectrometry; PALM, pollen-associated lipid mediator; PP, phytoprostanes.

Atopic diseases are characterized by a predominance of Th2-biased immune responses to environmental allergens (1). Allergen-specific Th2 cells are the key orchestrators of allergic reactions, initiating and propagating inflammation through the release of a number of Th2 cytokines such as IL-4, which regulates isotype switching to allergen-specific IgE (2), or IL-5, which recruits and activates eosinophils (3). Whereas the biology of Th2 cells in allergy is well understood, little is known about the mechanisms that control the initial Th2 polarization in response to exogenous allergens. Some studies suggest allergen-dependent mechanisms determined at the DC level due to particular attributes of the specific protein (4–6). Others suggest T cell–dependent (7) or individual (8, 9) factors leading to a predominance of the Th2 response.

C. Traidl-Hoffmann and T. Jakob contributed equally to this work.

DCs are pivotal in the initiation of adaptive immune responses (10). They produce IL-12 (one of the crucial Th1-polarizing cytokines) upon activation by pathogen-associated molecular patterns such as LPS (11) or by T cell–derived signals such as CD40 ligation (12). However, simultaneous presence of endogenous signals such as IL-10, TGFβ, corticosteroids, vitamin D_3, or PGE_2 can convert DCs from Th1- to Th2-skewing antigen presenting cells (13, 14). Recent studies also demonstrate that exogenous factors such as lipids produced by parasites can modulate DC function for the purposes of evading host immunity (15). These observations have generated a growing interest in defining how and which additional exogenous signals may regulate DC function in a fashion that may result in an altered generation of Th1- versus Th2-dominated immunity.

In the context of allergy, pollen grains have simply been regarded as allergen carriers, and little attention has been devoted to nonprotein compounds of pollen. However, individuals are rarely exposed to pure allergens, but rather to particles releasing the allergen such as pollen grains or pollen-derived granules (16–18). Notably, lipids are major components of pollen excine and exsudate (17). In addition, long chain unsaturated fatty acids in pollen, such as linolenic acid, serve as precursors for the biosynthesis of several plant hormones such as dinor isoprostanes, recently termed phytoprostanes (19–21). Phytoprostanes are formed nonenzymatically via autooxidation in plants and structurally resemble prostaglandins and isoprostanes in humans (22, 23). Recent results suggest that phytoprostanes might have an evolutionary ancient function in plant host defense (22, 23). Whereas the physiological role of phytoprostanes in the life cycle of plants is just beginning to emerge, virtually nothing is known about their effects on the human immune response in health and disease.

We demonstrated recently that pollen, under physiological exposure conditions, release not only allergens but also bioactive lipids that activate human neutrophils and eosinophils in vitro (24–26). Here we describe the ability of aqueous birch pollen extracts (*Bet.*-APE) to affect maturation and cytokine release of human DCs that results in an increased capacity to induce Th2 responses in naive T cells. By means of negative chemical ionization gas chromatography-mass spectrometry (NCI GC-MS) analysis of *Bet.*-APE, we demonstrate the presence of E_1-, F_1-, and A_1/B_1-phytoprostanes and show that E_1-phytoprostanes (similar to *Bet.*-APE) dose dependently inhibit IL-12 production and induce an increased Th2-polarizing capacity of human DCs. To the best of our knowledge, this represents the first study demonstrating that plant isoprostanes can affect the outcome of mammalian immune responses.

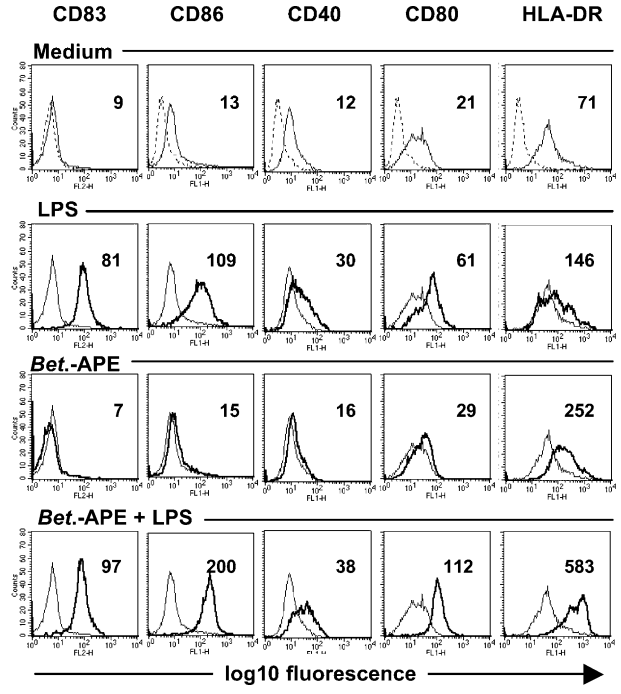

Figure 1. *Bet.*-APE effects on DC maturation. Immature DCs were left untreated (medium), stimulated with LPS (100 ng/ml) or LPS-depleted *Bet.*-APE (1 mg/ml), or simultaneously with both stimuli. Surface marker expression was analyzed after 24 h using flow cytometry. Medium control DCs (thin line) served as reference for all other culture conditions (bold line). Numbers indicate the net mean fluorescence intensity. Representative experiment of $n = 5$ using DCs from different nonatopic donors.

RESULTS

Induction of phenotypic and functional DC maturation by *Bet.*-APE

To investigate the impact of soluble factors released from pollen on the function of human DCs, immature monocyte-derived DCs were exposed to *Bet.*-APE and phenotypical and functional DC maturation was analyzed. Analysis of *Bet.*-APE by *Limulus* amebocyte lysate (LAL) test revealed substantial quantities of LPS (ranging from 10 to 500 EU/ml). Elution over polymyxin B columns allowed efficient removal of LPS (<0.05 EU/ml). LPS-depleted *Bet.*-APE was used for all subsequent experiments. Exposure of immature DCs to *Bet.*-APE alone induced an up-regulation of HLA-DR surface expression, whereas the remaining maturation markers (CD40, CD80, CD83, and CD86) remained unchanged (Fig. 1). When DCs were stimulated simultaneously with LPS plus *Bet.*-APE, the presence of *Bet.*-APE resulted in an additional up-regulation effect of CD80, CD86, and HLA-DR surface expression (Fig. 1). The change in surface marker expression was also reflected at a functional level when analyzing the allostimulatory capacity of DCs in MLR. Exposure of immature DCs to *Bet.*-APE alone resulted in an enhanced proliferative response of allogeneic naive T cells (Fig. 2, A and B). Simultaneous DC stimulation with *Bet.*-APE and LPS appeared to cause additive effects (Fig. 2, A and B). The effect of *Bet.*-APE on the allostimulatory activity was dose dependent (Fig. 2 B).

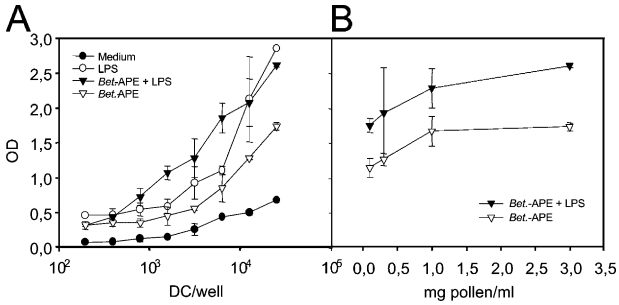

Figure 2. *Bet.*-APE induce DCs allostimulatory activity. (A) Immature DCs were left untreated (medium) or stimulated with LPS (100 ng/ml), *Bet.*-APE (3 mg/ml) alone, or together with LPS (100 ng/ml). After 24 h, DCs were analyzed for their capacity to induce T cell proliferation in naive allogeneic $CD4^+$ $CD45RA^+$ T lymphocytes (10^5/well). (B) DCs (2.5×10^4) stimulated with graded concentrations of *Bet.*-APE alone or together with LPS (100 ng/ml) were coincubated with naive T cells. T cell proliferation was measured by BrDU incorporation after 3 d of coculture. Results are given as mean OD ± SD of triplicate cultures and are representative of five independent experiments from different nonatopic donors.

Bet.-APE inhibits DC IL-12 production

Under control conditions (medium) DCs spontaneously released low levels of IL-12 (43.4 ± 18.4 pg/ml, $n = 6$), IL-6 (393 ± 280 pg/ml), TNFα (<78 pg/ml, $n = 6$), and IL-10 (11.8 ± 5.3 pg/ml, $n = 5$). LPS stimulation induced an upregulation of these cytokines 82-, 33-, 40-, and 29- fold, respectively. Interestingly, *Bet.*-APE when added simultaneously with LPS dose-dependently inhibited LPS-induced IL-12 p70 release, although it had no significant effect on basal IL-12 production (unpublished data). In contrast, LPS-induced IL-6, IL-10, and TNF-α release was not affected (Fig. 3 A). The inhibition of IL-12 p70 was not due to cytotoxic effects as determined by propidium iodide staining. *Bet.*-APE similarly inhibited the IL-12 p70 production when DCs were activated by CD40 ligation (unpublished data).

IL-12 is a heterodimeric cytokine, consisting of covalently bound p40 and p35 subunits (27). Distinct genes encode each subunit and each gene is independently regulated. To investigate the effects of *Bet.*-APE on IL-12 p40 and p35 mRNA expression DCs were stimulated with LPS in the presence or absence of increasing concentrations of *Bet.*-APE. LPS stimulation resulted in a strong induction of IL-12 p40 mRNA ($[2^{\{-\Delta\Delta CT\}}]$: 1,149 ± 518, $n = 3$) whereas IL-12 p35 mRNA was induced to a lesser degree ($[2^{\{-\Delta\Delta CT\}}]$: 364 ± 164, $n = 3$). Simultaneous addition of increasing concentration of *Bet.*-APE lead to a dose-dependent inhibition of IL-12 p40 mRNA expression (Fig. 3 B). In contrast, *Bet.*-APE stimulation seemed to enhance the LPS-induced IL-12 p35 mRNA (Fig. 3 B). These results suggest that *Bet.*-APE–dependent inhibition of IL-12 p70 release is likely to be regulated at the level of IL-12 p40 mRNA expression.

To rule out IL-10 as an autocrine inhibitor of IL-12 production (28) DCs were stimulated with LPS and *Bet.*-APE in the presence or absence of neutralizing anti–IL-10 mAb (10 μg/ml; R&D Systems). Although IL-10 neutralizing mAb restored the inhibition of IL-12 release induced by exogenous IL-10 (10 ng/ml; R&D Systems) back to normal, it did not restore IL-12 production inhibited by *Bet.*-APE (Fig. 3 C). To exclude effects of endogenous prostaglandins such as PGE_2, DCs were stimulated in the presence of the cyclooxygenase inhibitor indomethacin (25 μg/ml). Inhibition of DC cyclooxygenase did not reverse the inhibitory effects of *Bet.*-APE, suggesting that the observed inhibition was independent of endogenous prostaglandin production (Fig. 3 D).

Aqueous extracts of pollen grains from a variety of different plants such as alder, hazel, lilac, mapel, and mugwort displayed similar inhibitory activity on LPS-induced DC IL-12 production (Fig. 3 E), although IL-6 release was not significantly affected, suggesting that the observed immunomodulatory activity is not restricted to birch pollen but rather a more general phenomenon shared by pollen grains from different species.

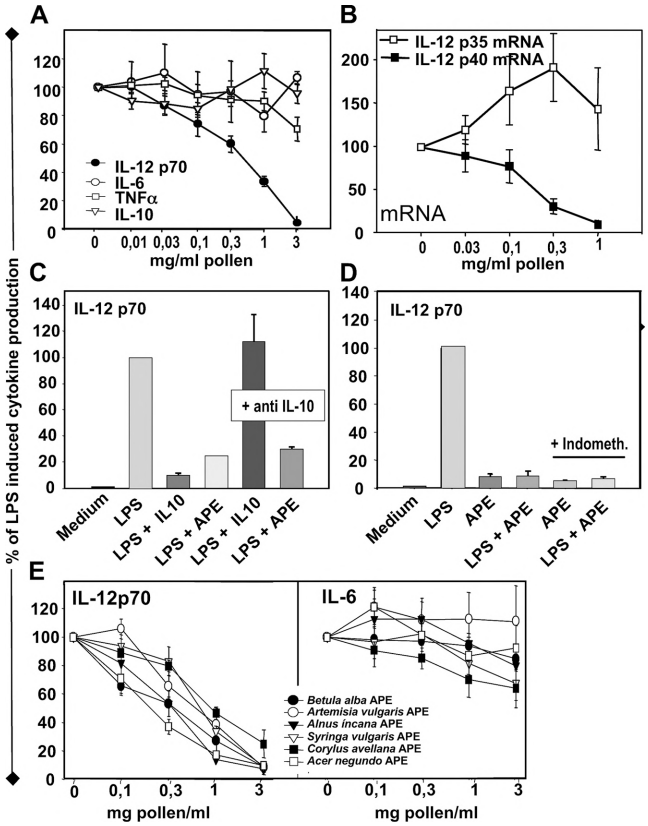

Figure 3. *Bet.*-APE inhibit DC IL-12 but not IL-6 production. (A) DCs were stimulated with LPS (100 ng/ml) in the presence of increasing concentrations of *Bet.*-APE. After 24 h IL-12 p70, IL-6, TNFα, and IL-10 concentrations were determined in culture supernatants. Data are presented as percentage of LPS-induced cytokine production in order to equalize donor specific variabilities (IL-12 p70 $\bar{x} = 3{,}599 \pm 1{,}446$ pg/ml, $n = 6$; IL-6 $\bar{x} = 13{,}069 \pm 7{,}358$ pg/ml, $n = 6$; TNFα $\bar{x} = 3{,}068; \pm 440$ pg/ml, $n = 5$; IL-10: $\bar{x} = 343 \pm 149$ pg/ml, $n = 5$). *Bet.*-APE reduce LPS-induced IL-12 p40 but not IL-12 p35 mRNA levels. (B) Immature DCs were stimulated for 6 h with LPS in presence of increasing concentrations of *Bet.*-APE. Quantitative IL-12 p40 and p35 mRNA analysis was performed. Data is shown as percentage of LPS-induced IL-12 p40 mRNA ($[2^{\{-\Delta\Delta CT\}}]$: 1,149 ± 518, mean ± SEM, $n = 3$) or IL-12 p35 mRNA ($[2^{\{-\Delta\Delta CT\}}]$: 364 ± 164, mean ± SEM, $n = 3$) expression. *Bet.*-APE–induced inhibition of DC IL-12 release is independent of autocrine IL-10 or endogeneous cyclooxygenase products. (C and D) DC stimulations were performed in the presence of IL-10–neutralizing antibodies (10 μg/ml; C) or a cyclooxygenase inhibitor (25 μM indomethacine; D). Data is presented as percentage (mean ± SEM) of IL-12 p70 production induced by LPS (C: IL-12 p70: $\bar{x} = 2{,}481 \pm 416$ pg/ml; $n = 4$; D: IL-12 p70 $\bar{x} = 4{,}126 \pm 326$ pg/ml; $n = 3$). APE from different species display similar effect on DC IL-12 and IL-6 production. (E) DCs were stimulated with LPS (100 ng/ml) in the presence of increasing concentrations of APE generated from different pollen species. After 24 h, IL-12 p70 and IL-6 levels were determined in culture supernatants (IL-12 p70 $\bar{x} = 1{,}477 \pm 426$ pg/ml, $n = 3$; IL-6 $\bar{x} = 10{,}776 \pm 1{,}463$ pg/ml, $n = 3$).

Bet.-APE exposure shifts DC polarizing capacity from Th1 to Th2

The inhibitory effects of *Bet.*-APE on DC IL-12 production prompted us to analyze the phenotype of primary T cell responses induced by DCs matured in the presence of *Bet.*-APE. Naive allogeneic T cells primed by LPS-matured DCs differentiated into Th1 lymphocytes with characteristic production of large amounts of IFN-γ and low levels of IL-4 (Fig. 4). In contrast, DCs activated by LPS in the presence of

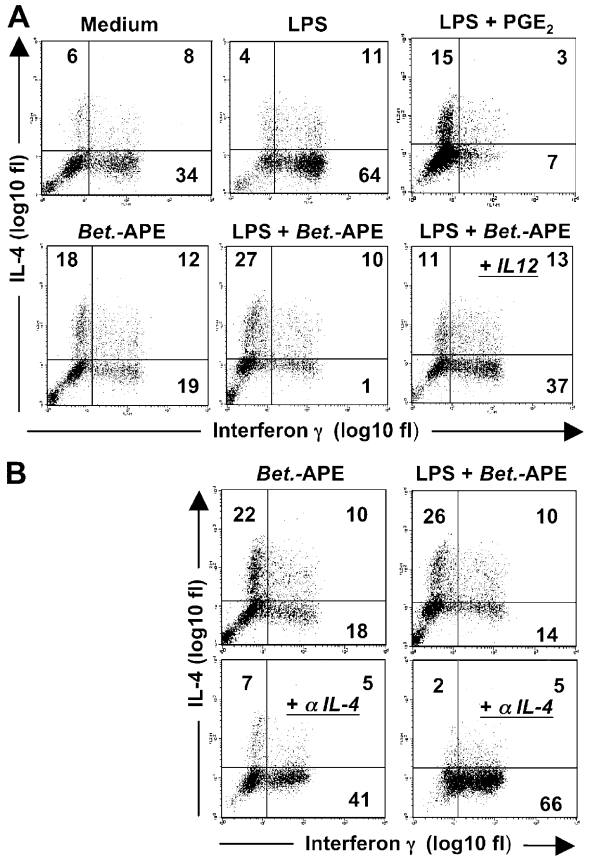

Figure 4. DCs matured in the presence of *Bet.*-APE display reduced Th1- and increased Th2-polarizing capacity. (A) DCs were left untreated or stimulated with *Bet.*-APE (3 mg/ml) in the presence or absence of LPS (100 ng/ml). After 24 h DCs were washed and cocultured with $CD4^+CD45RA^+$ allogenic T cells (DC/T cell ratio 1:4) that were expanded for 12 d in the presence of IL-2. T cell polarization was determined by analyzing intracellular IFN-γ and IL-4 accumulation via flow cytometry after restimulation with PMA and ionomycin in the presence of brefeldin A during the last 2 h of stimulation. To address, if the *Bet.*-APE–dependent Th2 polarization could be reverted by exogenous IL-12, hrIL-12 (10 ng/ml) was added at the beginning of the coculture of *Bet.*-APE/LPS-treated DCs and T cells. Representative experiment of $n = 3$–6 (compare Table I). To explore the role of IL-4 in the Th2 polarization induced by *Bet.*-APE–treated DCs, IL-4–neutralizing antibodies (10 μg/ml) were added at the beginning of the DC/T cell coculture. Representative experiment of $n = 3$.

Bet.-APE displayed a dramatically reduced capacity to induce IFN-γ–producing Th1 cells and a markedly enhanced capacity to induce IL-4–producing Th2 cells. The *Bet.*-APE–induced shift of a primarily Th1-dominated response to a primarily Th2-dominated response was comparable to that obtained under maximal Th2-polarizing condition, i.e., when DCs where stimulated with LPS in the presence of PGE_2 and neutralizing anti–IL-12 mAb was added at the beginning of the MLR (Fig. 4 and Table I). The *Bet.*-APE–induced shift from a Th1- to a Th2-dominated immune response could partially be restored, when exogenous IL-12 was added at the beginning of the DC–T cell coculture (Fig. 4), indicating that indeed inhibition of the DC IL-12 production by *Bet.*-APE plays a crucial role in the observed deviation of the immune response. However, addition of exogenous IL-12 (at concentration exceeding those measured in DC cultures) was not able to restore the response completely, suggesting that besides IL-12 other Th1-driving mediators may be inhibited by exposure of DCs to *Bet.*-APE. In contrast, when IL-4–neutralizing antibodies were added at the beginning of the T cell–DC coculture (Fig. 4 B) induction of Th2 cells was almost completely abrogated, demonstrating that the Th2-polarizing effect of Bet-APE–treated DCs was clearly IL-4 dependent.

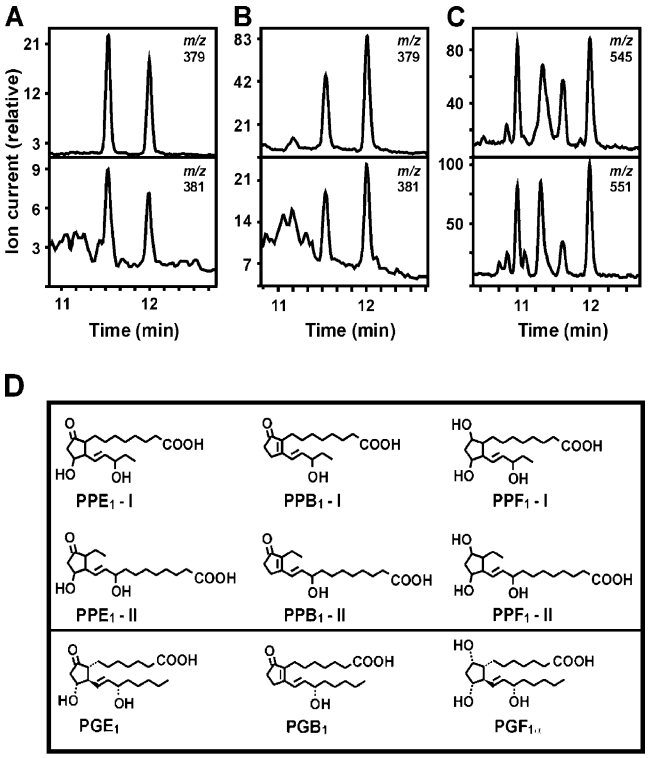

Figure 5. Analysis of phytoprostanes in *Bet.*-APE. Representative selected ion monitoring GC–NCI–MS traces of phytoprostanes from birch pollen extracts are shown. (A-C) PPE_1, PPA_1, and PPB_1 were extracted, purified, and analyzed as their corresponding pentafluorobenzyl ester, trimethylsilyl ether PPB_1 derivatives. (A) Endogenous PPE_1 derivatives are identified in the *m/z* 379 ion current chromatogram and quantitated against the $[^{18}O]PPE_1$ internal standard in the *m/z* 381 ion current chromatogram. (B) During sample processing, PPA_1 isomerize into the thermodynamically more stable PPB_1 isomers. Thus, the sum of endogenous PPA_1 and PPB_1 derivatives are identified in the *m/z* 379 ion current chromatogram and quantitated against the $[^{18}O]PPB_1$ internal standard in the *m/z* 381 ion current chromatogram. (C) Endogenous PPF_1 derivatives are identified in the *m/z* 545 ion current chromatogram and quantitated against the $[^{18}O]_3PPF_1$ internal standard in the *m/z* 551 ion current chromatogram. (D) Prostaglandin and phytoprostane structures. Prostaglandins E_1, B_1, and $F_{1\alpha}$ (PGE_1, PGB_1, and $PGF_{1\alpha}$, respectively) are derived from 8,11,14-eicosatrienoic acid via a cyclooxygenase pathway that is present in mammals but not in plants. E_1-, B_1-, and F_1-phytoprostanes (PPE_1, PPB_1, and PPF_1, respectively) are formed nonenzymatically via free radical intermediates. Phytoprostanes, derived from α-linolenic acid, occur in plants but not in mammals that lack this fatty acid. Two regioisomeric phytoprostanes (types I and II) of each phytoprostane family are present as racemates in plants due to their nonenzymatic formation.

Table I. *Bet.*-APE and E1-phytoprostanes induce Th2 polarization

DC stimulation	IL-4+[b]	IFN-γ+[c]	IL-4 + IFN-γ+[d]	IL-4/IFN-γ ratio[e]	*n*
Medium	13.8 ± 6.4	37.0 ± 3.3[g]	8.8 ± 0.4	0.36 ± 0.05	6
LPS	12.3 ± 2.4	59.2 ± 3.7[f]	8.3 ± 0.8	0.22 ± 0.05[f]	6
LPS + PGE_2[a]	38.8 ± 6.5	15.5 ± 4.2	9.7 ± 2.1	2.5 ± 0.18	6
LPS + *Bet.*-APE	24.3 ± 3.8[f,g]	25.8 ± 4.1[f,g]	8.0 ± 2.0	1.06 ± 0.18[f,g]	6
APE	22.3 ± 2.4	36.9 ± 2.01	9.7 ± 0.5	0.6 ± 0.09	6
LPS	15.7 ± 3.1	53.7 ± 4.3	10.5 ± 2.1	0.30 ± 0.17	3
LPS + PPE_1	33.3 ± 0.3[f,g]	36.7 ± 2.6[g]	14.3 ± 3.3	0.92 ± 0.06[f,g]	3
LPS + PPF_1	15.0 ± 0.5	58.7 ± 6.1	10.7 ± 0.3	0.27 ± 0.04	3
LPS + PPB_1-I	18.7 ± 3.5	55.3 ± 6.3	11.3 ± 1.2	0.38 ± 0.12	3
LPS + PPB_1-II	19.0 ± 2.6	58.0 ± 6.0	15.7 ± 2.8	0.35 ± 0.08	3

[a]To obtain a maximal Th2 polarization αIL-12 (10 mg/ml) was added at the beginning of the DC–T cell coculture.
[b]Percentage of IL-4–producing allogeneic CD4 T cells 12 d after activation by differently activated human DC (see Materials and methods; as determined by intracellular cytokine staining, mean ± SEM).
[c]Percentage of IFN-γ–producing allogeneic CD4 T cells.
[d]Percentage of double positive (IL-4 and IFN-γ) cells.
[e]Ratio of the percentage of IFN-γ– and IL-4–producing cells.
[f]$P < 0.05$ as compared to medium control.
[g]$P < 0.05$ as compared to LPS stimulation.

Bet.-APE contain substantial amounts of phytoprostanes

Previously it has been shown that various classes of prostaglandin-like compounds, the phytoprostanes, apparently occur ubiquitously in plants (23). Notably, exceptionally high levels of F_1-phytoprostanes (PPF_1) have been observed in organic extracts of birch pollen (21). In an attempt to identify potential candidates responsible for the observed effects of *Bet.*-APE, we quantified levels of phytoprostanes present in *Bet.*-APE by NCI GC-MS (Fig. 5, A–C and Table II). PPF_1 levels in pollen released in aqueous buffer (*Bet.*-APE) were 2.25 μg/g pollen. In addition, A_1/B_1- and E_1-phytoprostanes were detected in *Bet.*-APE. Interestingly, PPE_1 levels were found to be eightfold more abundant, whereas concentrations of PPA_1/B_1 were found to be threefold less abundant as compared with PPF_1 (Table II).

E_1-Phytoprostanes inhibit LPS-induced DC IL-12 p70 production and augment DC's capacity to induce Th2 responses. Various prostaglandins have been reported to modulate human DC function and cytokine profile (14, 29). The structural similarity of α-linolenic acid–derived phytoprostanes and prostaglandins prompted us to analyze whether phytoprostanes identified in *Bet.*-APE (PPE_1, PPF_1, and PPA_1/PPB_1) showed similar effects on human DCs. For this purpose DCs were activated by LPS in the presence or absence of phytoprostanes or PGE_2 over a wide range of concentrations (Fig. 6). As reported previously, PGE_2 dose dependently inhibited the LPS-induced IL-12 p70 release (14), whereas LPS-induced IL-6 production remained unchanged. PPF_1 and PPB_1 did neither affect the LPS-induced IL-12 p70 production nor the LPS-induced IL-6 production. However, PPE_1 markedly inhibited the LPS-induced IL-12 p70 production, without affecting the IL-6 production. The inhibitory effect of PPE_1 on DC IL-12 production was only observed when DCs were activated (e.g., by LPS) whereas in the absences of activation signals PPE_1 alone did neither modulate the basal IL-12 production, nor the outcome of the DC induced Th cell response. The inhibition of IL-12 release by PPE_1 was not due to cytotoxic effects, as determined by propidium iodide exclusion. In contrast to the effect on LPS-induced IL-12 production none of the phytoprostanes tested had any significant effect on LPS-induced DC maturation (unpublished data). In addition to

Table II. Concentrations of phytoprostanes in *Bet.*-APE

	Mol Wt	Concentration in *Bet.*-APE[a] (10 mg/ml)	Concentration[a] (μg/g pollen)	*n*
PPE_1	326	543.6 ± 41.1 nM	17.72 ± 1.34 μg/g	3
PPF_1	328	68.6 ± 1.5 nM	2.25 ± 0.05 μg/g	3
PPA_1/B_1	308	23.8 ± 3.5 nM	0.74 ± 0.11 μg/g	3

[a]Phytoprostanes were quantified in 10 mg/ml *Bet.*-APE by GC-NCI-MS (see Materials and methods).

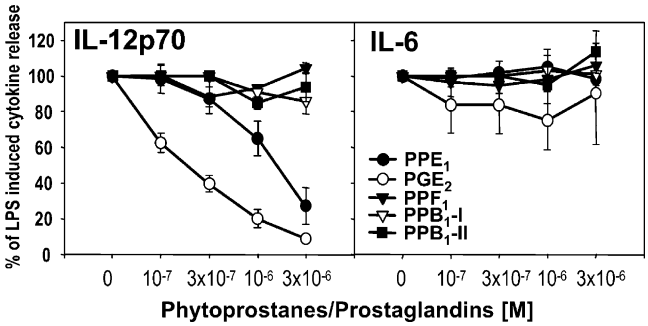

Figure 6. E_1-Phytoprostanes inhibit DC IL-12 production. Phytoprostanes (PP) identified in *Bet*-APE were analyzed with regard to their effect on DC cytokine production. DCs were stimulated with LPS (100 ng/ml) in the presence of different phytoprostanes (PPE_1, PPF_1, PPB_1-I, and PPB_1-II) in nontoxic concentrations (10^{-7} M -3×10^{-6} M). Data is presented as percentage (mean ± SEM) of IL-12 p70 (left) or IL-6 (right) production induced by LPS (IL-12 p70 $\bar{x}$ = 1,587 ± 397 pg/ml; IL-6 $\bar{x}$ = 21,214 ± 5,422 pg/ml; $n = 5$).

phytoprostanes, we recently demonstrated that *Bet.*-APE contain substantial quantities of monohydroxylated derivatives of α-linolenic and linoleic acid, such as 9- and 13-hydroxyoctadecatrienoate as well as 9- and 13-hydroxyoctadecadienoate (25, 26). Since some of these lipids have been suggested to inhibit the IL-12 production in human macrophages (30) we analyzed their effect on human DCs. Interestingly, none of these mediators (10^{-11}–10^{-5} M) lead to an inhibition of the LPS-induced IL-12 p70 release of human DCs (unpublished data). The PPE_1-dependent inhibition of DC IL-12 production prompted us to analyze the effects of various phytoprostanes on Th1–Th2 polarizing capacity of DCs. Presence of PPE_1 but not PPF_1 or PPB_1 during LPS-induced DC activation lead to the generation of DCs that displayed an increased capacity to induce Th2 polarization in naive T cells, as determined by intracellular cytokine staining (Table I).

DISCUSSION

Pollen grains are one of the most common inducers of allergic symptoms. Upon contact with mucosal surfaces of the upper respiratory tract, pollen grains rapidly release proteins/allergens into the aqueous phase. On the basis of a genetic susceptibility atopic individuals develop allergen-specific Th2-biased immune responses that ultimately lead to clinical manifestations of IgE-mediated hypersensitivity. Although the biology of Th2 cells in the effector phase of allergy is well understood, little is known about the mechanisms that control the initial Th2 polarization in response to exogenous allergens.

We recently demonstrated that pollen in addition to liberating protein allergens rapidly release various bioactive lipids into the aqueous phase (24–26). These pollen-associated lipid mediators (PALMs) were shown to stimulate and attract cells of the innate immune system, such as neurophil and eosinophil granulocytes (25, 26). Here, we describe the effect of PALMs on the activation and functional maturation of human DCs. In addition, we demonstrate that *Bet.*-APE and certain phytoprostanes identified in *Bet.*-APE modulate the function of human DCs in a fashion that results in a preferential induction of Th2-dominated adaptive immune responses.

Activation of DCs with LPS depleted by *Bet.*-APE (LPS under the detection limit of the LAL test) resulted in moderate DC activation as documented by selective up-regulation of HLA-DR surface expression. When DCs were stimulated simultaneously with LPS plus *Bet.*-APE, the presence of *Bet.*-APE resulted in an additional up-regulation of CD80, CD86, and HLA-DR surface expression. At a functional level *Bet.*-APE–induced DC maturation resulted in an enhanced allostimmulatory activity as demonstrated by enhanced proliferative responses of naive allogeneic T cells. In addition, *Bet.*-APE treatment induced a dose-dependent inhibition of the LPS or CD40L induced IL-12 p70 production of DCs, whereas IL-6, IL-10, and TNFα production was not impaired. Thus, water-soluble factors released from pollen grains are capable to selectively modulate various DC functions, including the inhibition of activation-induced IL-12 release from human DCs. The reduced IL-12 production was confirmed at mRNA level, demonstrating that regulation occurred predominantly at the level of IL-12 p40 rather than IL-12 p35.

Maturation of DCs is stimulated by factors signaling tissue danger such as microorganisms, dying cells, or proinflammatory cytokines. Recently, a variety of factors has emerged that can limit DC maturation. For example intracellular cAMP-elevating agents, such as PGE_2, inhibit IL-12 and TNFα and enhance IL-10 expression by LPS-stimulated DCs (14, 31). In contrast, IL-10, glucocorticoids, and vitamin D3 interfere with DC maturation as a whole by blocking the up-regulation of presenting and costimulatory molecules (13, 32, 33). *Bet.*-APE seemed to act independently of the above-cited mechanisms because its activity was not affected by indomethacin or neutralization of endogenous IL-10.

Recently a series of isoprostanes with the characteristic prostaglandin ring systems was discovered in plants and designated phytoprostanes (19). Phytoprostanes are formed via autooxidation, which is initiated by free radical attack of α-linolenic acid yielding a linolenate radical that readily oxidizes and cyclizes to two regioisomeric, prostaglandin G–like compounds (34). In vivo, PPG_1 may be either reduced to PPF_1 or converted to PPE_1, which itself may be dehydrated and isomerized to PPB_1 (19, 21).

In the present study we demonstrate for the first time that nonenzymatically formed phytoprostanes such as PPE_1, PPF_1, and PPB_1 are present in aqueous pollen extracts in nanomolar concentrations as identified and quantified by NCI GC-MS (19, 21). Levels of PPF_1 in organic extracts of birch pollen appear to be approximately 15 times more abundant in organic as compared with aqueous extracts (21). These differences might reflect different extraction efficiencies as well as varying concentrations in pollen from different sources. A survey of PPF_1 levels in fresh pollen from individual *betula pendula* L. trees and different birch species extracted with organic solvents revealed that levels vary greatly and range from 2 to 33 μg/g pollen (unpublished data). A similar variance is expected for PPE_1 levels. Phytoprostane levels in organic extracts reflect lipid peroxidation in pollen, and may only be of limited relevance for estimates of natural exposure levels on the mucus membranes. In contrast, analysis of phytoprostanes levels spontaneously released into the aqueous phase of the buffer used in this study, more closely mimics physiological exposure conditions.

PPE_1, PPF_1 and PPB_1 were tested in their capacity to modulate the IL-12 production of human DCs. Interestingly, only PPE_1 but not PPF_1 or PPB_1 inhibited the LPS or CD40-induced IL-12 production. Although *Bet.*-APE induced a functional and phenotypical maturation, none of the phytoprostanes tested had any significant effect on DC maturation (unpublished data). The modulatory effect of PPE_1 on DC IL-12 production and the ensuing T cell response was dependent on the presence of a maturation signal such as LPS or CD40 ligation. The receptors and signal transduction pathways involved in these mechanisms are currently under investigation.

It is generally accepted that DCs instruct the immune system to initiate an Ag-specific response by providing naive Th cells with signal 1 (TRC triggering) and signal 2 (costimulation). In addition, it has recently been suggested that immature DCs in peripheral non lymphoid tissue can adopt different Th1- or Th2-promoting effector function, depending on the tissue- and/or pathogen-type context of their activation (14). This DC-dependent component of the initial polarization of naive T cells (signal 3) was suggested to depend on pathogen-derived or -induced endogenous factors present in the local microenvironment at the time of antigen encounter. Our study demonstrates that this signal 3 can also be modulated by exogenous mediators such as phytoprostanes that are released from (under normal circumstances nonpathogenic) pollen grains upon contact with the airway mucosa.

Clearly, any extrapolation of these effects to the in vivo situation would partly depend on the expected concentration of pollen-derived lipids in the nasal or bronchial microenvironment. As demonstrated previously, concentrations of linolenic and linoleic acid in pollen are high (25, 26) and we assume that during pollen season the upper respiratory tract mucosa is exposed to biologically relevant concentration of various oxidized derivatives of these fatty acids. The effects of in vivo exposure to PALMs are currently under investigation.

Collectively, our data provide compelling evidence for the role of exogenous pollen-derived phytoprostanes in the decision-making process of DCs. We suggests that DCs that have been conditioned by PALMs, such as E_1-phytoprostanes will provide one of the initial signals driving the development and perpetuation of Th2-dominated immune response in pollen allergy.

MATERIALS AND METHODS

Reagents and Abs. Human rIL-4 was obtained from Promocell, human rGM-CSF from Essex, soluble CD40L (sCD40L) from Alexis. Purified LPS (*Escherichia coli* K235-derived LPS; <0.008% protein) was provided by Dr. Stephanie Vogel (University of Maryland, College Park, MD). FITC- or PE-conjugated anti–HLA-DR, anti-CD1a, anti-CD86, anti-CD80, anti-CD83, anti-CD1a, anti–IL-4, and anti–IFN-γ mAb were purchased from Becton Dickinson, anti-CD4 and anti-CD45RA microbeads from Miltenyi Biotech.

Preparation of *Bet.*-APE. Birch pollen grains (*Betula alba* L.) were obtained from Allergon. *Bet.*-APE were generated by incubation of pollen grains in RPMI 1640 (30 mg/ml) for 30 min at 37°C followed by centrifugation (20 min at 3,345 *g*) and sterile filtration (0.2 μm; 24). LPS was measured by LAL assay (Cambrex Bio Science). To deplete LPS, *Bet.*-APE were eluted over polymyxin B columns (Pierce Chemical Co.) leading to LPS concentrations below the detection limit of the assay (<0.05 EU/ml). LPS-depleted *Bet.*-APE was used for subsequent experiments.

Monocyte-derived DCs. Healthy, nonatopic blood donors were characterized by screening for total and specific IgE for common allergens as described recently (25). Monocyte-derived DCs were prepared from peripheral blood of healthy individuals, as described recently (35). In brief, adherent PBMC (>90% pure $CD14^+$ cells) were cultured at 10^6 cells/ml in RPMI 1640 supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies) supplemented with 10% FBS, 500 U/ml human rGM-CSF (Essex Pharma) and 500 U/ml human rIL-4 (Promocell; complete DC medium) at 37°C under 5% CO_2. At day 5 cells (>95% $CD1a^+$, $CD14^-$) were harvested and recultured in complete DC medium for 24 h at 37°C with or without indicated stimuli in the presence or absence of LPS (100 ng/ml) or soluble CD40L (1 μg/ml; Alexis) followed by addition of a cross-linker (1 μg/ml). Aliquots of DC culture supernatants were assayed for IL-12 p70, IL-6, IL-10, and TNFα by two site ELISAs using antibodies from BD Biosciences as described previously (36).

Flow cytometry of DCs. Surface expression of DC maturation markers was analyzed using multicolor flow cytometry as described recently (37). In brief, DCs (either untreated or stimulated for 24 h with LPS in the presence or absence of pollen extracts or phytoprostanes) were harvested, washed, and suspended in cold PBS containing 5% FCS and 0.02% NaN_3, and then serially incubated with saturating concentrations of FITC-conjugated mAb, and PE-conjugated mAb. Matched isotype control mAb were used in control samples. Stained cells were analyzed using a FACS Calibur flow cytometer equipped with CellQuest software (Becton Dickinson). Propidium iodide-permeable (nonviable) cells were excluded from analysis.

MLR. Human $CD4^+$, $CD45RA^+$ T cells were purified from nonadherent PBMC from healthy nonatopic donors using magnetic cell sorting column separators with anti-CD4 and anti-CD45RA microbeads (Miltenyi Biotec). Differently stimulated DCs (24 h) were washed and cocultured with magnetic cell sorting–purified allogeneic naive $CD4^+$, $CD45RA^+$ T cells (10^5 cells/well) in complete RPMI with 5% human serum. Cell proliferation was quantified using a BrdU cell proliferation ELISA (Amersham Biosciences). To analyze T cell polarization, DC/T cell cocultures were incubated in a 96-well plate at a DC/T ratio of 1:4 and T cells were subsequently expanded in 24-well plates in medium supplemented with IL-2 (20 U/ml; Chiron Corp.). LPS-activated DCs (24 h) were harvested, washed twice, and used for priming to generate Th1-polarized T cells ("Th1 control"). Th2-polarized T cells ("Th2 control") were generated by using DCs that were activated (24 h) with LPS in the presence of PGE_2 (10^{-6} M; Qbiogene). In addition, neutralizing anti-IL-12 mAb (10 μg/ml; BD Biosciences) was added at the beginning of the DC/T cell coculture in order to generate a maximal Th2 polarization.

Intracellular cytokine staining. After 12 d of culture, T cells were restimulated with PMA (20 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 6 h and examined for intracellular IFN-γ and IL-4 accumulation. To prevent cytokine secretion, Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the final 2 h. T cells were fixed (2% paraformaldehyde), permeabilized (0.5% saponin), and stained with FITC-conjugated mouse anti–IFN-γ and PE-conjugated rat anti–IL-4 mAb or isotype-matched control mAb and analyzed by flow cytometry as described previously (38).

Quantitative mRNA analysis. Total RNA was extracted from purified DCs after a 6-h incubation with the indicated stimuli using peqGOLD RNAPure buffer (Peqlab). RNA was reverse transcribed using random hexamer primers (Roche). PCR reactions for IL-12 p40 and p35 (Assay on Demand; Applied Biosystems) were run on an ABI PRISM 7700 Sequence Detection System device (Applied Biosystems) using the following program: 10 min at 94°C followed by 40 cycles of 15 s at 95°C, and 60 s at 55°C. 18 s RNA served as housekeeping gene.

Analysis of PPE_1, PPA_1/B_1, and PPF_1 by NCI GC-MS. Aqueous extracts (20 ml) of birch pollen (200 mg) were treated with 10 ml of a saturated NaCl solution in water containing 0.05% butylated hydroxytoluene (wt/vol), 20 mg of triphenylphosphine, 0.2 ml of 1 M citric acid, and isotopically labeled phytoprostane standards. Phytoprostanes were extracted with diethyl ether, purified, derivatized, and analyzed by NCI GC-MS as described previously (21).

Preparation of PPE_1, PPB_1, and PPF_1. Racemic E_1- and F_1-phytoprostanes were prepared by autoxidation of α-linolenic acid and purified as

described previously (19, 21). B_1-Phytoprostanes were obtained by base-catalyzed isomerization of E_1-phytoprostanes (21).

Statistic. Student's paired *t* test was used to compare groups and ratios of IL-4– or IFN-γ–producing T cells induced by differently stimulated DCs. $P < 0.05$ was considered significant.

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