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Pollen-derived low-molecular weight factors inhibit 6-sulfo LacNAc$^+$ dendritic cells' capacity to induce T-helper type 1 responses

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Summary

Background Evidence is accumulating that the pollen exsudate contains an array of non-allergenic, pro-inflammatory and immunomodulatory substances acting on the innate and adaptive immune system. In this context, pollen-associated E_1-phytoprostanes (PPE_1) were shown to licence human monocyte-derived dendritic cells for T-helper type 2 (Th2) polarization of naïve T cells.

Objective This study aims at analysing the impact of pollen-associated lipid mediators on cytokine secretion and maturation of 6-sulfo LacNAc$^+$ dendritic cells (slanDCs), the most abundant native dendritic cell (DC) in human peripheral blood, and further dissecting the biologically active substance(s) within aqueous pollen extracts.

Results Aqueous birch pollen extracts dose-dependently inhibited the lipopolysaccharide (LPS)-induced IL-12 p70 production, while the levels of IL-6 remained unaffected. PPE_1 inhibited secretion of both IL-12 p70 and IL-6. Aqueous pollen extracts, but not PPE_1 or F_1-phytoprostanes significantly reduced the LPS-induced surface expression of the maturation markers CD80, CD83, CD40 and CCR-7, an effect that was independent of proteins and that was still present in a 3 kDa cut-off fraction of the pollen extract. These effects were observed irrespective of the atopy status of the donors. Finally, slanDCs exposed to aqueous pollen extracts were impaired in eliciting an IFN-γ response in naïve CD4$^+$ T cells.

Conclusion Our data show that slanDCs, a subset of human blood DCs with constitutively high potency to induce Th1 responses, are susceptible to the Th2 polarizing effect of low molecular weight, non-protein factors derived from pollen.

Keywords allergy, IL-12, maturation, pollen-associated lipid mediators, slanDC

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Introduction

In the past years, research focussed on the identification of allergenic proteins from pollen. However, not until recently it became clear that pollen grains contain eicosanoid-like substances, the pollen-associated lipid mediators (PALMs), which are rapidly released in a humid milieu and act in multiple ways on resident cells, creating a micro-milieu favouring allergic immune responses [1]. Some PALMs act as chemoattractants for polymorphonuclear leucocytes and eosinophils [2, 3], others like the E_1-phytoprostanes (PPE_1) modulate dendritic cell (DC) function leading to T-helper type 2 (Th2) polarization of naïve T cells, foremost by inhibiting the DCs ability to produce the key Th1-cytokine IL-12 p70 [4]. However, aqueous birch pollen extracts (APE) mediate multiple effects on DCs which cannot be attributed to PPE_1 [5], like intracellular cAMP formation and the induction of a chemokine receptor expression pattern similar to that induced by prostaglandin E_2 (PGE_2), licensing the DC to Th2 attraction [6].

While it becomes increasingly clear that different DC subsets play distinct roles during the course of allergic disorders like atopic eczema [7] or asthma [8, 9], the origin of tissue DCs remains elusive. Our previous studies on the effects of PALMs on DC function were conducted with human monocyte-derived dendritic cells (moDCs).

*Contributed equally to this work.

A major human blood DC subset, 6-sulfo LacNAc$^+$ DCs (slanDCs), were first discovered by Schaekel and colleagues [10, 11]. The frequency of slanDCs in human peripheral blood mononuclear cells (PBMC) is about 1–2%, which makes them the most abundant DCs in human blood [12]. SlanDCs belong to the myeloid lineage and express high levels of FcγRIII (CD16) [11], which distinguishes them from other blood DCs such as CD1c$^+$ MDCs and CD123$^+$ PDCs. They undergo spontaneous maturation if cultured in the absence of erythrocytes [13]. Once matured, they are characterized by a high potential to produce pro-inflammatory cytokines such as IL-12 p70 and TNF-α, and to prime naïve T cells to become Th1 cells [14]. They are found in the T cell area of inflamed human tonsils and in inflamed ileal mucosa of Crohn's disease patients and might be the cell-type responsible for the high serum TNF-α levels in bacterial sepsis [15]. SlanDCs have also been detected in psoriatic [13] and eczematous skin lesions (K. Schaekel, unpublished data).

In this study we addressed the question whether phytoprostanes or other low molecular weight pollen-derived factors modify the function of slanDCs. Our data give first evidence that low molecular weight factors interfere with multiple functions of slanDCs, and that this seems to be a general mechanism not dependant on atopy status.

Material and methods

Subjects

Healthy, non-atopic blood donors were characterized by screening for total and specific IgE to common allergens as recently described [4]. Non-atopic donors (aged 20–51 years) were characterized by low total serum IgE-levels (< 50 IU/mL), lack of specific IgE against the common allergens screened for, and a negative clinical history of allergic disease. Atopic donors were characterized by higher total serum IgE (295±181 IU/mL), sensitization against *Phleum pratense* and *Betula* pollen (specific IgE 8.9±2.6 and 42.5±15 IU/mL, respectively) and a positive history of seasonal allergic rhinitis. All volunteers were without medication for at least 15 days before blood sampling. The ethical committee of the Technische Universität Munich approved the study and volunteers were enroled in the study after written informed consent.

Phytoprostanes, reagents and kits

PPE_1 and PPF_1 (each as a 1 : 1 mixture of two regio-isomeres) were prepared by autoxidation of α-linolenic acid and purified as described before [16]. Mouse anti-human antibodies used for flow-cytometry were IgG_1-FITC, CD3-FITC, CD80-FITC, CD40-FITC, IgG_1-PE, CD16-PE, CD83-PE, CCR-7-PE, IgG_1-PE-Cy5, CD86-PE-Cy5, IgG_1-APC, HLA-DR-APC, IgG_{2a}-APC (all from BD Pharmingen, Heidelberg, Germany). For staining of slanDCs, goat $F(ab')_2$ Fragment Anti-Mouse IgM (μ)-FITC was used (Beckman Coulter, Krefeld, Germany). This secondary antibody detects cells, which are still labelled with anti-M-DC8 primary antibody from the isolation procedure. *Escherichia coli* ultra-pure lipopolysaccharide (LPS) was purchased from InvivoGen (Toulouse, France). ELISA kits used were Human IL-12 p70 and human IL-6 matched pair (BD Pharmingen).

Generation of APE

Aqueous pollen extracts were generated from pollen grains of white birch (*Betula alba* spec., Sigma Aldrich, Taufkirchen, Germany) as described previously [4]. LPS was extracted using AffinityPak^{TM} Detoxi-Gel^{TM} Endotoxin Removing Gel columns (Pierce, Rockfort, IL, USA). Endotoxin levels were below detection limit (< 0.5 U/mL) as determined by LAL assay (Lonza, Basel, Switzerland). The concentrations of APE given in the text and figures refer to the original amount of pollen that was extracted (e.g. 3 mg/mL = extract of 3 mg pollen/mL medium).

Proteinase K digestion of APE

Proteins were removed from the extracts by digestion with 1 μg/mL Proteinase K (Sigma Aldrich) at 37 °C for 2 h. The enzyme was heat inactivated for 10 min at 80 °C, and degradation of proteins was verified by SDS-PAGE and coomassie staining.

Fractionation of APE with molecular weight cut-off membranes

Three kilodalton cut-off fractions of APE were generated using YM-3 Microcon Centrifugal Filter Devices (Millipore, Schwalbach, Germany), following the manufacturer's manual. After the separation, both filtrate ($APE^{<3\,kDa}$) and concentrate ($APE^{>3\,kDa}$) were restored to the initial volume and frozen at −80 °C until further processing.

Isolation of slanDCs

PBMCs were prepared by density gradient centrifugation and labelled with anti-M-DC8 antibody and Rat anti-mouse IgM MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany). SlanDCs were isolated from labelled PBMCs by positive selection using an AutoMACS^{TM} separator (Miltenyi Biotech). Directly following isolation, purity and expression of the markers CD80, CD86, CD83, CD40, HLA-DR, CCR-7, CD206 and FcεRI were determined by 3-colour flow-cytometry on a FACScalibur (Becton Dickinson). Cells were 91±6% pure M-DC8$^+$ CD16$^+$ slanDCs, contaminanting cells being CD3$^+$ T cells (data not shown).

Stimulation of slanDCs

SlanDCs were seeded into 96-well flat-bottom plates at a densitiy of 0.75×10^6 cells/mL DC medium (RPMI 1640, 10%FBS, 2 mmol/L L-glutamine, 20 μg/mL gentamycin, 500 μmol/L 2-mercaptoethanol) and stimuli were added to the following final concentrations: APE 3.0 mg/mL, PPE_1 5×10^{-7} M, PPF_1 5×10^{-7} M. Unstimulated slanDCs served as control. After 6 h (37 °C, 5% CO_2), LPS was added to a final concentration of 100 ng/mL to appropriate wells. The pre-incubation period of 6 h in the absence of LPS is necessary for the survival of the cells. After 24 h (37 °C, 5% CO_2), cells were harvested, stained and subjected to FACS analysis. Dead cells as determined by propidium iodide staining were excluded from analysis.

For the determination of cytokine secretion, APE or PPE_1 were added to the DC culture at different concentrations. Medium served as control. After 6 h at 37 °C, 100 ng/mL LPS was added to appropriate wells, and cells were incubated for a total of 24 h (37 °C, 5% CO_2). Supernatants were collected and subjected to cytokine ELISA. Cells were stained with propidium iodide and subjected to FACS analysis.

Allogenic MLR

SlanDCs were cultured for 6 h in the absence or presence of APE, PGE_2, PPF_1 or PPE_1, washed and LPS (100 ng/mL) was added to appropriate wells. Allogenic $CD4^+$ $CD45RA^+$ T cells were isolated using the $CD4^+$ $CD45RA^+$ Naïve T Cell Isolation Kit (Miltenyi Biotech) and added to slanDCs at a T cell : DC ratio of 10 : 1. After 10 days, cells were restimulated with PMA/Ionomycin in the presence of monensin and brefeldin A for 6 h. Cells were fixed, permeabilized and stained with anti IFN-γ-FITC and anti IL-4-PE (BD Pharmingen). Intracellular cytokine expression of T cells was determined by flow cytometry.

Statistical analysis

To reveal statistically significant differences between treatment groups, two-tailed Wilcoxon test for paired samples was used. *P*-values $<0.05^*$ or $<0.01^{**}$ were considered to indicate significance.

Results

Aqueous pollen extracts inhibit the LPS-induced secretion of IL-12 p70 but not of IL-6

To investigate whether APE impact on the cytokine secretion of slanDCs, supernatants of slanDCs matured in the presence of LPS plus different concentrations of APE were analysed for the release of IL-12 p70 and IL-6. 24 h supernatants of unstimulated slanDCs did not contain detectable levels of IL-12 p70 and IL-6 (Figs 1a–d), and APE treatment alone did not induce the production of either cytokine (data not shown). LPS-stimulation enhanced both IL-12 and IL-6 (Figs 1a–d) (IL-12: 2500±1000 pg/mL; IL-6: 15 000±2000 pg/mL). IL-10 was not detectable under any condition tested (data not shown). APE dose-dependently decreased the production of IL-12 p70 (Fig. 1a), while the secretion of IL-6 remained unaffected (Fig. 1b).

PPE_1 inhibit the LPS-induced production of IL-12 and IL-6

We next investigated whether pollen-derived PPE_1 might exert an inhibitory effect on the production of IL-12 p70, as in the case of moDCs. PPE_1 dose-dependently inhibited the secretion of both IL-12 p70 (Fig. 1c) and IL-6 (Fig. 1d). PPE_1 alone did not induce cytokine secretion of slanDCs, and IL-10 was not detected under either condition (data not shown).

APE, but not PPE_1 or PPF_1 inhibit the LPS-induced up-regulation of the surface markers CD80, CD83, CD40 and CCR-7

Freshly isolated slanDCs showed low expression of T cell costimulatory markers CD80 and CD40, of CD83 and of the chemokine receptor CCR-7. After 24 h, the expression of CD80, CD83 and CCR-7 was slightly induced even in the absence of stimulus, while CD40 expression remained low. Addition of APE or PPE_1 or $PPF_1$to the culture medium did not significantly change the baseline expression of CD80, CD83, CD40 or CCR-7, while after stimulation with LPS all four markers were strongly induced (Figs 2a–d). APE significantly inhibited the LPS-induced surface expression of CD80, CD83, CD40 and CCR-7 (Figs 2a–d). PPE_1, at 5×10^{-7} M, which corresponds to the PPE_1 concentration in APE [4], decreased the induced expression of CD83, an effect that did not reach statistical significance. All other markers remained unaffected by PPE_1. PPF_1 did not have any significant effect on the induced expression of either marker (Figs 2a–d).

Freshly isolated slanDCs expressed considerable amounts of CD86 and HLA-DR. Both molecules were markedly induced after 24 h even in the absence of a stimulus (Figs 2e and f). Although none of the stimuli induced any statistically significant change in baseline expression, the spontaneous up-regulation of CD86 and HLA-DR was slightly decreased in the presence of APE and PPE_1, while PPF_1 did not inhibit spontaneous maturation (Figs 2e and f). In the presence of LPS, both CD86 and HLA-DR were induced approximately threefold. When APE or PPE_1 were present, the LPS-induced up-regulation of CD86 and HLA-DR was decreased to some extent, an effect, however, that was not statistically significant. Again, the presence of PPF_1 did not alter the expression of either marker significantly (Figs 2e and f).

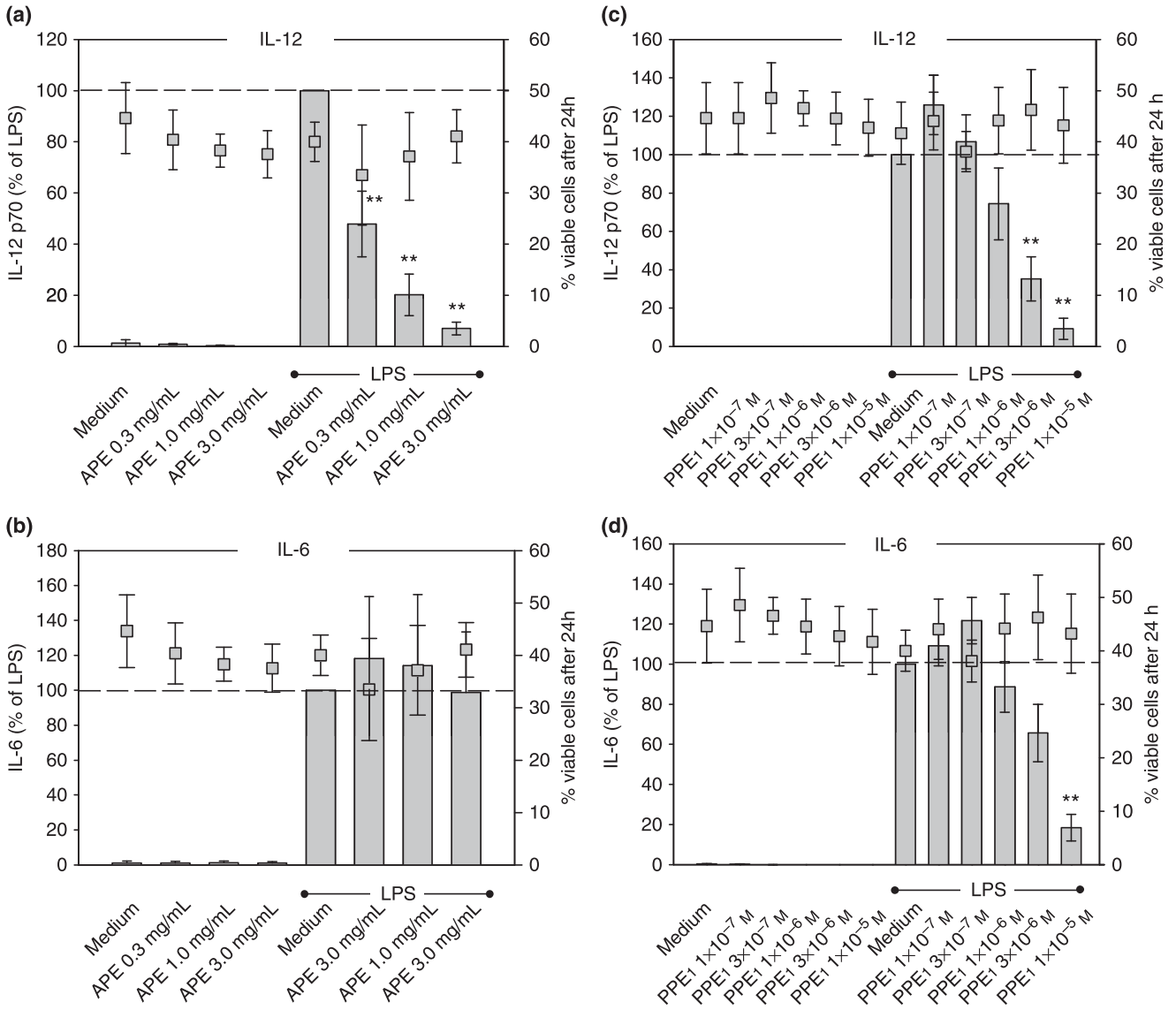

Fig. 1. Aqueous pollen extracts and E_1-phytoprostanes (PPE_1) inhibit cytokine production of 6-sulfo $LacNAc^+$ dendritic cells (slanDCs). SlanDCs were matured with 100 ng/mL *Escherichia coli* lipopolysaccharide (LPS) in the absence or presence of different concentrations of aqueous birch pollen extracts (APE; a and b) or PPE_1 (c and d). After 24 h, cells were harvested, stained with propidium-iodide and subjected to FACS analysis to determine the percentage of survived cells (filled squares). Supernatants were obtained and analysed for the presence of IL-12 p70 (a and c) and IL-6 (b and d) by ELISA. Graphs are expressed as the percentage of mean LPS-induced cytokine secretion (IL-12: 2500±1000 pg/mL; IL-6: 15 000±2000 pg/mL). Depicted are mean values of 6 (a and b) and 10 (c and d) independent experiments performed with cells from different donors±SEM. $^{**}P<0.01$, two-tailed Wilcoxon's test.

Inhibitory effect of APE on the LPS-induced up-regulation of costimulatory molecules is not lost after proteinase K treatment of extracts

Since the inhibitory effect of APE on the induced up-regulation of the maturation markers CD80, CD83, CD40 and CCR-7 did not seem to be mediated by PPE_1 or PPF_1, we hypothesized that instead of PALMs, pollen-derived protein(s) might mediate this effect. We thus digested the pollen extracts with proteinase K, a carboxy peptidase degrading proteins released from pollen into the aqueous phase. We then analysed whether the digested APE still inhibited the LPS-induced expression of the maturation markers. Interestingly, the inhibitory effect of APE digested with proteinase K was indistinguishable from the effect of untreated APE (Fig. 3).

Inhibition of LPS-induced IL-12 production and up-regulation of maturation markers depends on molecules smaller than 3 kDa

To test whether the effects would still be present in a low molecular weight fraction of APE, pollen extracts

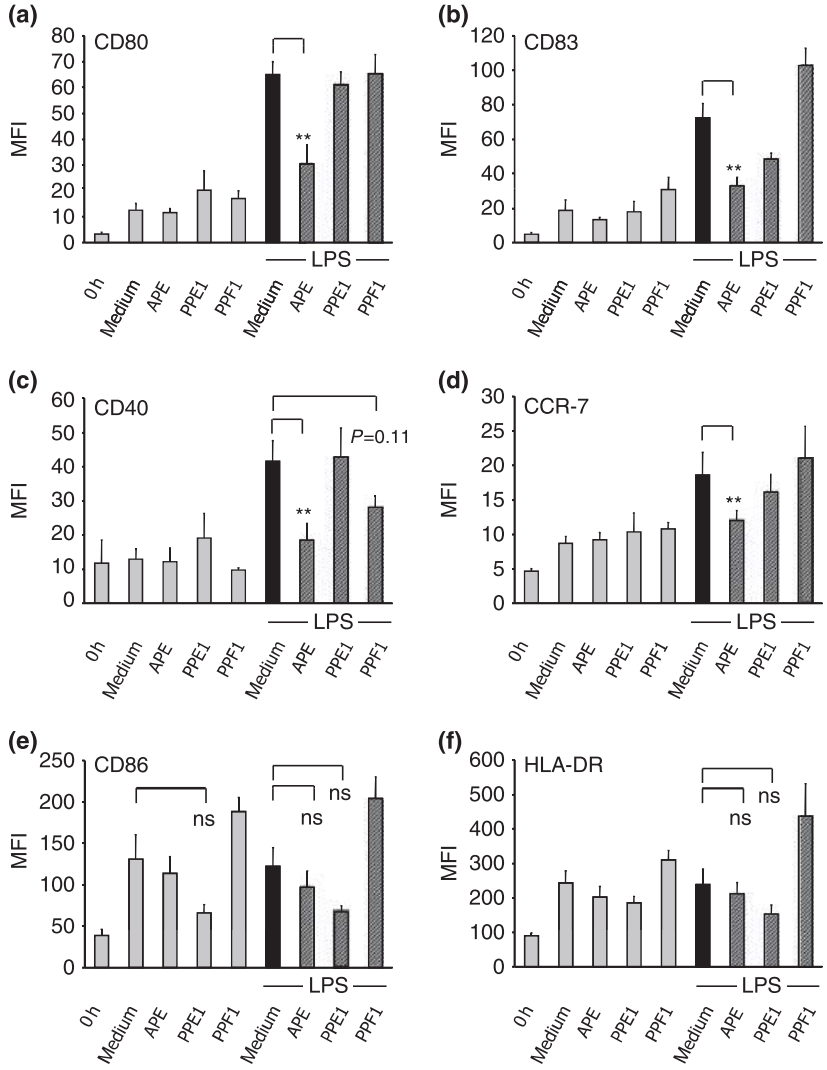

Fig. 2. Aqueous pollen extracts inhibit up-regulation of the maturation markers (a) CD80, (b) CD83, (c) CD40, (d) CCR-7, (e) CD86 and (f) HLA-DR. 6-sulfo LacNAc$^+$ dendritic cells were matured for 24 h with lipopolysaccharide (LPS) in the absence or presence of aqueous birch pollen extracts (APE, 3 mg/mL) or E_1-phytoprostanes (PPE_1) and PPF_1 (5×10^{-7} M). Cells were then harvested and subjected to three-colour FACS analysis. Data are expressed as mean fluorescence intensity (MFI). Depicted are mean values±SEM of 15 independent experiments performed with cells of different non-atopic donors. Under each condition, mouse IgG_1 served as isotype control, and the MFI of the isotype controls were subtracted from the MFI of specifically stained cells, respectively. $^{**}P<0.01$, two-tailed Wilcoxon's test.

were separated by a 3 kDa cut-off filter, and total APE (APE^{total}), concentrate (enriched for molecules over 3 kDa – $APE^{>3\,kDa}$) and filtrate ($APE^{<3\,kDa}$) were analysed for their ability to inhibit LPS-induced IL-12 production of slanDCs (Fig. 4). Notably, $APE^{<3\,kDa}$ was more effective than $APE^{>3\,kDa}$. We then tested the different fractions for their capacity to inhibit the LPS-induced slanDC maturation (Fig. 5). Both fractions were effective in blocking up-regulation of CD80 and CD40, but the effect was clearly more pronounced in the low molecular weight fraction. CD83 and CCR-7 were only inhibited significantly by $APE^{<3\,kDa}$, while the $APE^{>3\,kDa}$ was inefficient.

SlanDCs exposed to APE are impaired in Th1 cell induction

To elucidate whether the effects of APE on slanDC cytokine production and maturation might impact on T-helper cell polarization, we performed allogenic MLR with slanDCs matured in the presence or absence of APE or phytoprostanes (Fig. 6). SlanDCs matured for 6 h in the absence of stimulus were potent inducers of IFN-γ but did not induce IL-4 in naïve allogenic CD4$^+$ T cells. In contrast, slanDCs matured for 6 h with APE displayed a markedly reduced capacity to induce IFN-γ, while IL-4 production was induced in a very small fraction of T cells.

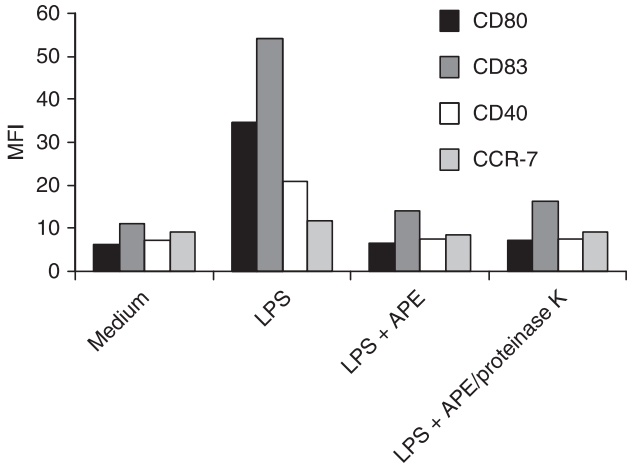

Fig. 3. Aqueous pollen extracts digested with proteinase K inhibit up-regulation of maturation markers. Aqueous birch pollen extracts (APE) were digested with proteinase K, followed by heat-inactivation of the enzyme. Then, 6-sulfo LacNAc$^+$ dendritic cells were stimulated with APE in the presence of lipopolisaccharide (LPS). After 24 h, expression levels of the maturation markers CD80, CD83, CD40 and CCR-7 were determined by flow cytometry. Data are expressed as mean fluorescence intensity (MFI). Under each condition, mouse IgG_1 served as isotype control, and the MFI of the isotype controls were subtracted from the MFI of specifically stained cells, respectively. Depicted is one representative experiment of three performed.

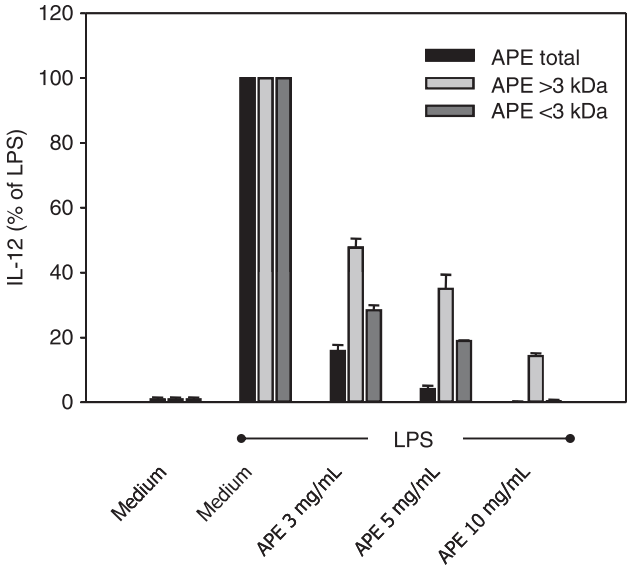

Fig. 4. The inhibitory effect of aqueous pollen extracts is retained in a low molecular weight fraction. Aqueous birch pollen extracts (APE) were filtered using 3 kDa cut-off membranes and filtrate (APE$^{<3\,kDa}$) as well as concentrate (APE$^{>3\,kDa}$) were restored to their original volume with PBS. 6-sulfo LacNAc$^+$ dendritic cells were incubated with the different fractions for 24 h in the absence or presence of lipopolysaccharide (LPS). Supernatants were analysed for the presence of IL-12 p70 by ELISA. Results are mean±SEM of three independent experiments.

Maturation in the presence of PPE_1 or PPF_1 did not have any influence on cytokine profile in ensuing T cells. Maturation of slanDCs with LPS lead to an increased potential to induce T cells' IFN-γ as compared with medium-treated slanDCs (24.0±6.0% vs. 10.8±0.4% IFN-γ-positve cells; mean±SEM of 3 independent experiments). When APE was present for the first 6 h of incubation, the T cell IFN-γ response was reduced slightly as compared with medium (4.9±0.5% vs. 10.8±0.4% IFN-γ-positve cells). SlanDCs exposed to APE for 6 h and then stimulated with LPS induced significantly less IFN-γ in T cells than slanDCs stimulated with LPS alone (7.6±1.4% vs. 24.0±6.0%). Also some – if only few – IL-4 positve cells were detectable. As in the absence of LPS, phytoprostanes did not shift the cytokine ratio significantly (Fig. 6).

The effect of APE on slanDCs is independent of atopy status of the donor

We finally analysed whether the effects of APE on slanDC maturation depended on the sensitization status of the donor (Table 1). Directly following isolation, slanDCs of atopic and non-atopic subjects expressed comparable levels of CD80, CD83, CD40 and CCR-7. After stimulation with LPS, up-regulation of maturation markers occurred in cells of both groups, and APE inhibited the LPS-induced up-regulation of the markers CD80, CD83, CD40 and CCR-7, independent of the atopy status (Table 1). Expression of CD86, HLA-DR, CD206 and FcεRI was not regulated by APE in either group (Table 2).

Discussion

Our study provides convincing evidence that low molecular weight components from birch pollen inhibit secretion of IL-12 p70 from LPS-matured slanDCs but do not affect IL-6 secretion. However, in contrast to moDCs [4], PPE_1 inhibited both the secretion of IL-12 p70 and of IL-6 in slanDCs. The observed discrepancy between the two DC subtypes might be explained by differential receptor employment or -expression.

Besides their effect on cytokine secretion, APE, PPE_1 and PPF_1 differentially influence the LPS-induced maturation of slanDCs. The maturation-induced up-regulation of the T cell costimulatory molecules CD80 (B7-2) and CD40, of the DC-specific marker CD83 and of the chemokine receptor CCR-7 were potently inhibited in the presence of APE. This effect was not mediated by the two major pollen-associated phytoprostanes, PPE_1 and PPF_1, which prompted us to ask whether proteins, possibly pollen-derived proteases might be responsible, e.g. by clipping the respective surface molecules. This effect is well described for house dust mite allergen Der p 1 [17, 18]. The inhibitory effect of APE, however, was still observed after digestion of APE with proteinase K. Furthermore, it was

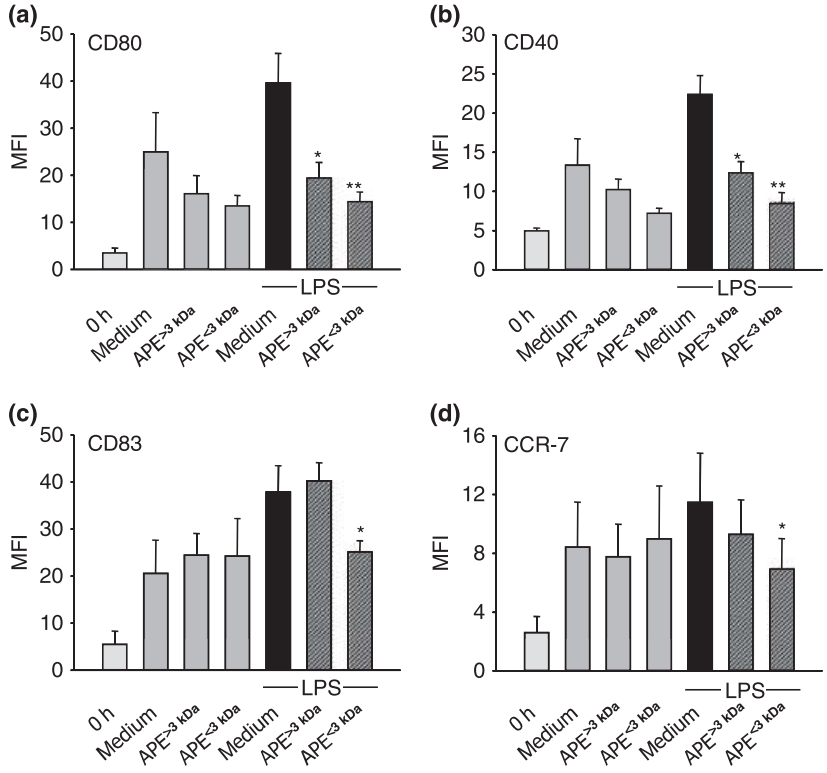

Fig. 5. A low molecular weight fraction of aqueous pollen extracts inhibits maturation of 6-sulfo LacNac dendritic cells. Cells were analysed for the expression of maturation markers CD80, CD83, CD40 and CCR-7 by flow cytometry. Data are expressed as mean fluorescence intensity (MFI) ±SEM. MFIs of isotype controls were subtracted. Depicted are mean values of four (CD40, CCR-7) or five independent experiments (CD80, CD83). $^{*}P<0.05$, $^{**}P<0.01$, two-tailed Wilcoxon's test.

Table 1. Aqueous pollen extracts inhibit the maturation of slanDCs of non-atopic as well as atopic donors

	CD80		CD83		CD40		CCR-7	
	Atopic	Non-atopic	Atopic	Non-atopic	Atopic	Non-atopic	Atopic	Non-atopic
$t=0$	2.8±0.2	3.0±0.9	3.9±1.1	4.6±1.4	9.6±0.8	4.4±2.0	4.3±0.8	4.3±0.7
Medium	13.4±11.3	9.5±5.5	12.3±12.9	9.5±3.3	14.2±7.8	13.4±8.1	5.6±1.6	8.8±3.8
APE	13.3±4.4	13.1±6.3	10.0±6.4	12.0±4.4	10.3±2.8	10.2±4.6	6.5±2.4	8.3±1.8
LPS	69.6±16.0	70.0±20.6	62.3±39.6	60.4±21.3	78.2±33.8	53.0±23.5	16.4±7.9	16.1±7.1
LPS+APE	34.8±16.4**	33.0±25.3**	31.6±14.6**	31.0±15.7**	24.1±24.9**	23.2±17.6**	8.9±5.4**	11.1±3.8**

Shown are mean MFI±SD of 15 (non-atopic) and 5 (atopic) independent experiments performed with cells from different donors. $^{**}p<0.01$, two-tailed Wilcoxon's test.

APE, aqueous birch pollen extracts; LPS, lipopolysaccharide; slanDCs, 6-sulfo LacNAc^{+} dendritic cells.

Table 2. Markers CD86, HLA-DR, CD206 and FcεRI are not regulated by aqueous pollen extracts

	CD86		HLA-DR		CD206		FcεRI	
	Atopic	Non-atopic	Atopic	Non-atopic	Atopic	Non-atopic	Atopic	Non-atopic
$t=0$	29.1±14.9	24.6±5.0	84.4±46.4	90.5±22.1	6.5±3.9	6.1±1.3	9.4±4.7	13.7±5.5
Medium	46.7±16.7	68.9±27.1	173.1±36.8	217.7±108.0	7.3±3.4	11.2±5.2	17.0±10.6	26.5±11.5
APE	70.1±8.2	91.8±33.1	154.3±32.4	202.2±109.3	10.0±4.7	15.0±6.0	13.6±7.5	31.7±25.9
LPS	64.7±21.2	75.8±17.5	161.3±10.0	184.7±89.7	8.5±3.9	13.7±5.5	14.0±6.6	25.4±9.9
LPS+APE	51.5±27.5	72.0±24.1	120.4±51.4	206.4±103.7	12.2±10.5	12.6±4.2	16.0±8.0	26.5±14.2

APE, aqueous birch pollen extracts; LPS, lipopolysaccharide; slanDCs, 6-sulfo LacNAc^{+} dendritic cells.

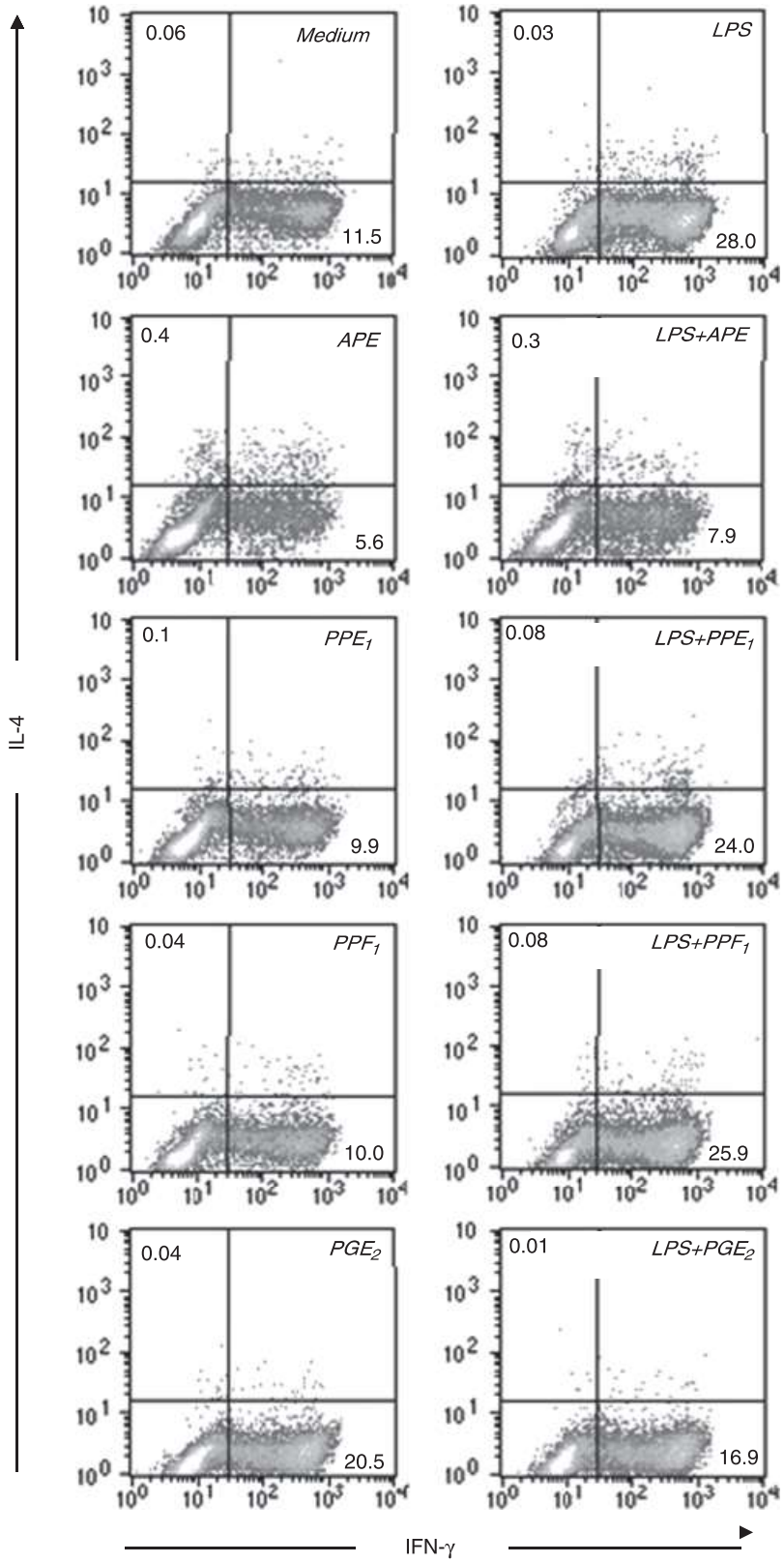

Fig. 6. Aqueous pollen extracts inhibit IFN-γ production of naïve $CD4^+$ T cells in MLR. Freshly isolated 6-sulfo $LacNAc^+$ dendritic cells were pre-matured either in the absence of stimulus (medium) or in the presence of indicated stimuli [Aqueous birch pollen extracts (APE): 3 mg/mL, E_1-phytoprostanes (PPE_1), PPF_1, prostaglandin E_2: 10^{-6} mol/L]. After 6 h, the cells were washed and naïve allogenic $CD4^+$ T cells were added at a DC : T cell ratio of 1 : 10. Lipopolysaccharide (LPS) (100 ng/mL) was added to half of the sample wells. After 10 days, cells were restimulated with PMA/Ionomycin and IFN-γ and IL-4 were determined by intracellular cytokine staining. Shown is one representative experiment of three performed. MFI, mean fluorescence intensity.

retained in a low molecular weight fraction of APE. Although we cannot exclude the involvement of a protein or low-molecular peptide, taken together, our results rather suggest the involvement of a non-protein factor, possibly an as yet unidentified lipid. In aqueous solution, lipids might be associated with proteins, which might explain the finding that although the inhibitory activity was strongest in the low molecular weight fraction of APE, both $APE^{<3\,kDa}$ and $APE^{>3\,kDa}$ exhibited some activity.

In monocytes and DCs, the expression of multiple co-stimulatory molecules is down-regulated by the cytokine IL-10 [19, 20]. However, we were not able to detect IL-10 in supernatants of slanDC cultures under any stimulus tested, which argues against IL-10 as regulatory mediator in our experimental setup.

In our hands, slanDCs pre-matured for 6 h in the absence of stimulus were potent inducers of IFN-γ-producing T cells, while hardly any IL-4-producing T cells were detectable. Pre-incubation of slanDCs with APE lead to strong inhibition of the T cell IFN-γ response. Surprisingly, pre-stimulation of slanDCs with PPE_1 (10^{-6} mol/L), although moderately effective in inhibiting IL-12 production, did not significantly shift the ensuing T cell IFN-γ/IL-4 ratio. This might indicate that instead of PPE_1, other low molecular components such as steroid hormones, flavonoids or other plant secondary metabolites mediate the observed modulation of slanDC function. There are several reports demonstrating that flavonoid compounds can impair DC maturation and/or cytokine production via inhibition of NF-κB and/or MAPK pathways [21–24]. Also, so called 'danger-associated molecular patterns' (DAMPs), e.g. nucleotides, have been implicated as potent modulators of DC function (reviewed in Willart and Lambrecht [25]).

In our last set of experiments we analysed whether the effects of APE on slanDC maturation depend on the sensitization status of the donor. The experiments were all conducted over a period of 3 months (January–March) when *Betula* pollen counts in the air can be estimated to be low [26]. All atopic donors were sensitized against *Betula* pollen, and *Betula*-APE was used for all stimulations. We found that the effect of APE on slanDC maturation was similarly pronounced in cells of atopic and non-atopic donors. It has been shown before that maturation in response to toll-like receptor ligands does not differ between DCs generated from monocytes of atopic and non-atopic donors [27]. Our data are in agreement with this report, because we did not observe statistically significant differences between slanDCs of atopic and non-atopic donors – neither in response to LPS nor to LPS plus APE-stimulation.

$CD16^+$ monocytes have been described as migratory DCs [28]. SlanDCs co-expressing CD16 might therefore play a role in immune surveillance in the skin and mucosa, which makes them potential targets for pollen-derived factors. High numbers of slanDCs have been found in inflamed ileal mucosa of Crohn's disease patients and in psoriasis vulgaris lesions [13, 15]. Both Crohn's disease and psoriasis are strongly Th1-dominated disorders. It is tempting to speculate that slanDCs might also be present in chronic atopic dermatitis lesions, where Th1-mediated inflammation dominates. Especially when the natural barrier of the skin is disrupted, PALMs and other pollen-derived adjuvant factors might gain access to epidermal and subepidermal tissue and impact local DC function. To date nothing is known about direct effects of PALMs on DCs in the tissue. We know, however, that exposure of atopic dermatitis skin to whole pollen grains in patch tests result in a substantial increase in epidermal $CD1a^+$ and $CD1b^+$ DC numbers [29], documenting that pollen-derived factors (possibly also PALMs) influence the DC compartment in an inflammatory condition *in vivo*.

In summary, we demonstrate for the first time that non-protein, small molecular weight substances from pollen can modulate the function of a native human DC subset on the level of cytokine production, co-stimulation and ensuing T cell response. These findings strongly suggest that PALMs – or other immunomodulatory factors from pollen – impact skin circulating or infiltrating DCs.

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