

Original article

Adjuvant activity of pollen grains

Background: The induction of an immune response to a biologically inert soluble protein requires an adjuvant. Here we have examined whether intact grains of pollen display such adjuvant effect, accounting for the immunogenic activity of pollen protein allergens that are devoid of intrinsic pro-inflammatory/adjuvant property.

Methods: Human monocyte-derived dendritic cells (DCs) were cultured with intact grains of grass or ragweed pollen for 48 h. The state of DCs maturation was analyzed by FACS and their cytokine production by ELISA. T cell priming activity of DCs was examined in co-cultures with naïve cord blood-derived CD4⁺ T cells.

Results: Contact with grains of pollen induced a distinct maturation program in immature DCs. Pollen up-regulated the expression of CD54, CD80, CD83, CD86, HLA-DR, CCR7, and CD40 on DCs. Moreover, CCR5 expression was up-regulated by pollen but suppressed by LPS. In sharp contrast to LPS-stimulated DCs, pollen-treated DCs did not produce cytokines [interleukin (IL)-10, IL-12, tumor necrosis factor (TNF)- α] but retained the ability to secrete high levels of these cytokines upon stimulation with soluble CD40 ligand and interferon (IFN)- γ . Pollen-primed DCs strongly stimulated the proliferation of allogeneic naïve CD4⁺ T cells and promoted their development into effector cells producing high levels of IL-5 and IL-13 together with moderate levels of IFN- γ and IL-4.

Conclusion: Intact grains of pollen induce activation and maturation of DCs *in vitro*. Similar mechanisms may be effective *in vivo*, suggesting that pollen grain is not only an allergen carrier but also acts as an adjuvant in the induction phase of the allergic immune response.

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Allergic diseases such as rhinitis or extrinsic asthma represent a major health problem of most modern societies, with significant economical consequences resulting from the reduced productivity of affected patients, as well as healthcare costs (1, 2). These diseases are caused by aberrant Th2 immune responses directed against protein Ags (also known as allergens) rapidly released by commonly inhaled small particles. Whereas the mechanisms whereby ongoing Th2 responses against these protein Ags lead to clinical asthma and rhinitis are understood in increasing detail, the early events involved in elicitation of the immune response or allergic sensitization are still unclear. Perhaps this is because the majority of studies conducted to date on the induction of immune or allergic responses to airborne allergens have relied on the utilization of purified allergen proteins (3, 4). However, we do not inhale pure protein Ags, but rather the small particles on which they are carried, such as pollen grains, their starch particles or other exogenous particulate materials (5, 6).

Pollen is one of the most frequent airborne allergens from the natural environment, leading to seasonal rhinitis

in about 10–25% of the population (7, 8). However, healthy nonatopic individuals develop low grade and noninflammatory immune responses to pollen Ags following natural exposure. Pollen allergens are apparently very immunogenic as they induce in both healthy and allergic individuals the same number of pollen-specific CD4⁺ T cells. Although the latter recognize the same epitopes of the protein Ags, they differ by their cytokine production profile (9, 10).

The mechanisms leading to the induction of immune responses to innocuous antigens such as the proteins released by pollens are unknown. Studies in animal models on the immune response to biologically inert proteins such as ovalbumine (OVA), deposited on the airway mucosa have demonstrated the obligatory role of the adjuvants. No airway inflammatory response was generated in mice that had been sensitized with LPS-depleted OVA, whereas antigen-specific immune responses were induced in the presence of LPS with low and high doses inducing Th2 or Th1 responses, respectively (11). It has been suggested that the proteolytic activity of some purified allergens may account for

their immunogenic activity by enhancing their trans-epithelial delivery and/or promoting local inflammation (12, 13). Der p1, the major allergen of house dust mite, is a cysteine protease that has been extensively investigated for its allergenic activity (12). No such proteolytic or intrinsic adjuvant activity has been described for pollen proteins. However, *in vitro* studies have shown that crude aqueous pollen extracts can directly recruit and activate polymorphonuclear neutrophils and eosinophils, inducing chemotaxis and cytokine secretion (14, 15).

Here we further explore the adjuvant activity of intact pollen grains and examine their ability to activate immature dendritic cells (DCs) and to influence their capacity to prime naïve T cells.

Materials and methods

Dendritic cell preparation and culture conditions

Peripheral blood mononuclear cells were isolated by density gradient centrifugation of heparinized blood from healthy volunteers with Lymphoprep (Nycomed). Monocytes were enriched by cold aggregation, followed by T cell depletion. To generate iDCs monocytes were cultured for 1 h in RPMI 1640 with 10 mM Hepes, 2 mM L-glutamine, 100 IU penicillin, and 100 µg/ml streptomycin. Nonadherent cells were removed, and after washing adherent cells were cultured for 6 d with GM-CSF (25 ng/ml) and interleukin (IL)-4 (25 ng/ml) in complete RPMI 1640 medium supplemented with 10% FCS. Mature DCs (mDCs) were generated after stimulation of iDCs (0.5×10^6 /ml) in complete RPMI medium containing 10% FCS with LPS (10 ng/ml) or with monocyte-conditioned medium (10%), prepared as described previously (16). In preliminary experiments different concentrations of pollen grains (5, 10, 25, 30 and 50 µg/ml; kindly provided by J. Robillon, Pasteur Institute, Paris, France) were analyzed for the ability to induce CD86 expression on iDCs and a plateau was reached for ragweed as well as grass pollen at 30 µg/ml. Purified protein (AgE; kindly provided by R. Esch, Greer Labs, NC, USA) from ragweed pollen was tested (10 µg/ml) for some experiments. Some cultures were done in RPMI medium containing polymyxin B (10 µg/ml) (17). In some experiments, mDCs were washed, counted, and restimulated for 48 h with 1 µg/ml sCD40-L (kindly provided by R. Armitage, Seattle) and 500 U/ml interferon (IFN)- γ .

Purification of human Naïve T cells and DC-T cell co-cultures

Naïve CD4⁺ T cells were isolated from umbilical cord blood of healthy neonates as described (18). Briefly, mononuclear cells were obtained by centrifugation on Lymphoprep and cell preparation was enriched in T cells by E-rosetting. CD4⁺ T cells were obtained by Easy-Sep procedure according to the manufacturer's instructions. Primary MLRs were conducted in 96-well U-bottomed tissue culture plates.

Flow cytometric analysis

The phenotypes of iDCs and mDCs were determined by direct staining with FITC-CD86 (ID), PE-CD83 (ID), PE-CD54 (ID), FITC-HLA-DR, PE-CCR5 for 30 min at 4°C. A three step staining

was performed for CCR7: unlabeled CCR7, followed by biotinylated goat anti-mouse IgG + IgM (1/500; Biosource International, Camarillo, CA, USA) and later stained with streptavidin-PE (Becton Dickinson BD Biosciences, Mississauga, ON, Canada). For intracytoplasmic staining, monensin was added for last 5 h of culture; cells were fixed for 10 min with 4% paraformaldehyde and permeabilized with 0.1% saponin.

Cytokine measurements

Interleukin-12 p70 release was assessed by a two-site sandwich ELISA using mAb 20C2 as the capture mAb and HRP-coupled mAb 4D6 as detection probe. Both mAbs were generously provided by Dr M. Gately (Hoffmann-La-Roche). The sensitivity of the assay was 6 pg/ml tumor necrosis factor (TNF)- α , IL10, IFN- γ , IL-5, and IL-4 release were assessed by two-site sandwich ELISA as previously described (19). The sensitivity of the assays was 50 pg/ml for these cytokines. IL-13 was measured by sandwich ELISA using rat anti-IL-13 mAb (clone 5A2; American Type Culture Collection) as capture mAb and a polyclonal rabbit anti-IL-13 Ab (Accurate Chemicals, Westbury, NY, USA) as a detecting probe. All the measurements were performed in duplicate.

Thymidine incorporation

Thymidine incorporation was assessed by adding 1 µCi/well ³H-thymidine (Amersham) during the last 6 h of the culture. Triplicate cultures were then harvested onto glass-fiber filters, and the radioactivity was counted using liquid scintillation.

Statistical analysis

Student's paired *t* test, nonparametric ANOVA and Mann-Whitney (nonparametric) tests were used to determine the statistical significance of the data. A threshold value of *P* < 0.05 was considered to be statistically significant.

Results

Contact with intact grains of pollen induces a maturation program in DCs

Upon stimulation with bacterial products, iDCs undergo several phenotypic and functional changes, a process known as DC maturation and leading to the development of fully competent antigen-presenting cells (20). To study the effect of grains of pollen on DC maturation, 30 µg/ml of pollen (ragweed or grass) was added to cultures of immature monocyte-derived DCs for 48 h and phenotype analysis was performed. The expression of CD54, CD80, CD83, CD86 and HLA-DR was strongly increased on DCs exposed to grains of pollen, with levels slightly lower than those obtained by exposure of DCs to LPS (Fig. 1A) or monocyte-conditioned medium (data not shown). In contrast, the effect of pollen on CD40 expression was much less pronounced. Purified protein from pollen (AgE) did not affect the expression of co-stimulatory molecules on immature DCs (data not shown). Three series of observations excluded the possibility that the effects of pollen were due to endotoxin contamination.

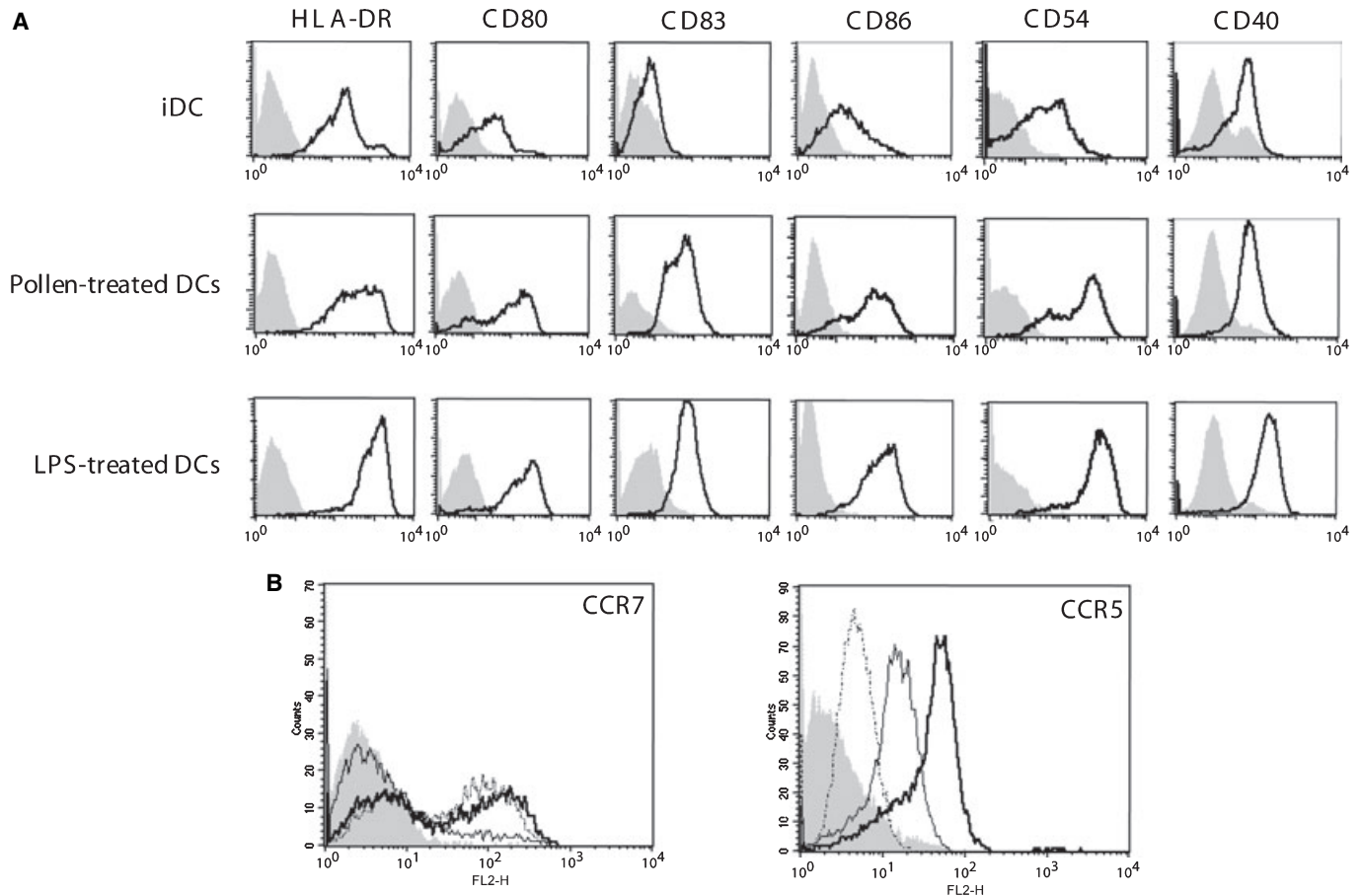


Figure 1. Contact with intact grains of pollen induces the expression of a mature phenotype in monocyte-derived DCs. (A) Pollen grains (30 $\mu\text{g/ml}$) or LPS (10 ng/ml) were added to the cultures for 48 h and the expression of co-stimulatory molecules was analyzed by FACS. Filled histograms indicate respective isotype controls. (B) CCR7 and CCR5 expression on iDCs (thin line), DCs treated with pollen grains (thick line) or LPS (dotted line) after 48 h of co-culture. Filled histograms indicate isotype controls. Results from one representative experiment are shown.

Firstly, inclusion of polymyxin B in the cultures did not affect the pollen-induced DCs maturation (17). Secondly, pollen grains were as active in HB101 serum-free medium as in complete culture medium, containing-LPS binding proteins. Thirdly, the effects of pollen were contact-dependent and not detected in the supernatant fluids of pollen suspensions.

A hallmark of mature DC is the down-regulation of the chemokine receptor CCR5 and the up-regulation of CCR7 (21, 22). This receptor switch is instrumental for emigration of maturing DCs from the peripheral tissues and their correct positioning in secondary lymphoid organs. The exposure to pollen grains caused up-regulation of CCR7 (MFI for CCR7 was 96 ± 11 for iDCs and 150 ± 19 and 131 ± 18 for pollen-treated and DC-LPS, respectively). Unexpectedly, pollen promoted up-regulation of CCR5 on DCs (Fig. 1B), whereas as predicted LPS treatment caused down-regulation of CCR5. This observation further militates against the possibility that the effects of pollen grains were caused by contaminating LPS.

Contact with intact grains of pollen induces the secretion of low levels of cytokines

The secretory activity of DC is of major importance to initiate, amplify, and orientate the immune response. Cytokine production was measured in culture supernatants of DCs treated for 48 h with pollen. Unlike LPS-matured DCs, DCs treated with pollen were unable to produce IL-12. Treatment with pollen induced the production of IL-10 and TNF- α , although five and six times less than in LPS-matured DCs, respectively (Fig. 2; 121 ± 66 in DC-pollen vs 633 ± 196 pg/ml in DC-LPS for IL-10 and 225 ± 66 in DC-pollen vs 1364 ± 448 pg/ml for TNF- α).

We next examined whether pollen may influence the LPS-induced production of TNF- α , IL-10 and IL-12, as the balance between these cytokines is of major importance in the APC activity and Th-polarizing function of DCs. Addition of pollen to LPS-treated DCs had no effect on their cytokine production profile (data not shown).

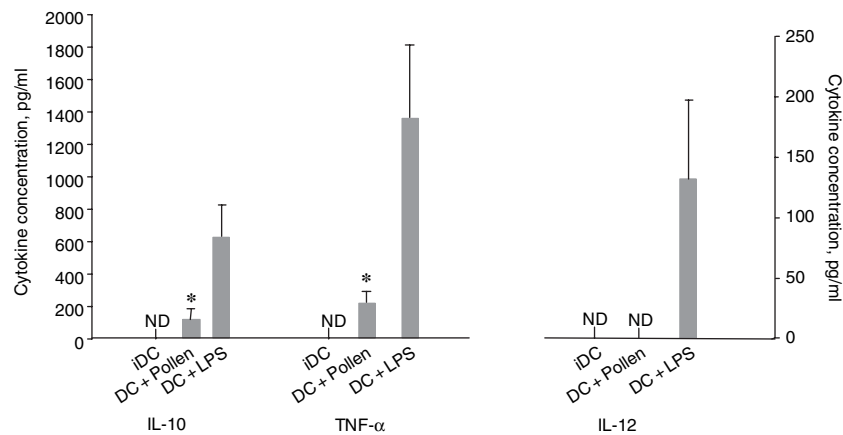


Figure 2. Cytokine production by pollen-treated DCs. 0.5×10^6 /ml monocyte-derived iDCs were treated for 48 h with grains of pollen (30 μ g/ml) or LPS (10 ng/ml). Culture supernatants were tested by ELISA for IL-10, IL-12p70, and TNF- α . *Significantly different compared with cells treated with LPS. Mean $\pm$ SEM of eight experiments. ND, not detected.

Collectively, the data indicate that upon contact with grains of pollen DCs become semi-mature in that they produce little or no cytokines and display only some phenotypic markers of mature DCs (23).

Cytokine production capacity of pollen-primed dendritic cells

To examine the cytokine production capacity of pollen-primed DCs, iDCs were cultured for 48 h with pollen, monocyte-conditioned medium or LPS, washed and re-stimulated for another 48 h with sCD40-L and IFN- γ in

order to mimic interaction with activated T cells. As seen in Fig. 3, pollen-treated DCs produced much higher levels of IL-10, IL-12 and TNF- α than DCs pretreated with monocyte-conditioned medium or LPS. The latter produced much less cytokines at re-stimulation than during primary stimulation, a finding consistent with the concept of DC exhaustion (24). In that context, unlike monocyte-conditioned medium- and LPS-matured DCs, pollen-treated DCs were not 'exhausted' and did not lose their ability to respond to secondary stimulation. Note that the response of pollen-primed DCs to CD40 stimu-

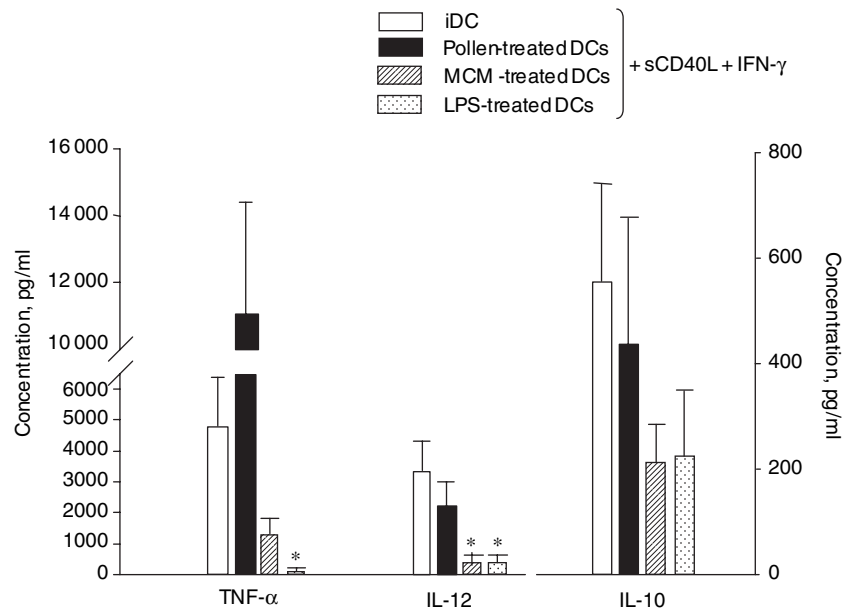


Figure 3. Cytokine production capacity of pollen-primed DCs. iDCs were cultured for 48 h with pollen grains, monocyte-conditioned medium (MCM) or LPS, washed and re-stimulated for another 48 h with sCD40 L (1 μ g/ml) and 500 U/ml IFN- γ (to mimic T cell interactions) and cytokines were measured in the supernatants of the cultures. *Significantly different compared with unprimed DCs (iDC). Mean $\pm$ SEM of six experiments.

lation is similar to that of iDCs, a finding consistent with the notion of semi-mature DCs (Fig. 3).

Pollen-treated DCs promote proliferation and differentiation of naïve T cells into high IL-13 and IL-5 producers

Pollen-treated DCs were much better than iDCs in inducing the proliferation of naïve allogeneic CD4⁺ T cells. Their stimulatory activity was comparable with that of LPS-treated DCs (Fig. 4).

We next examined whether pollen-treated DCs had polarized naïve T cell development into Th1, Th2 or regulatory T cells (Treg). To this end, umbilical cord blood-derived naïve CD4⁺ T cells were co-cultured for 7 d with iDCs, pollen-treated DCs and LPS-treated DCs at stimulator/responder ratios of 1 : 10. After 7 d of co-culture, cells were washed and stimulated for 48 h with anti-CD3 immobilized on mitomycin C-treated CD32/B7.1 L cells. Pollen-treated DCs were significantly better at inducing IL-5, IL-13 than iDCs (Fig. 5A). However, they did not differ from LPS-treated DCs. It is of note that the production of IFN- γ was not affected by the type of DCs. Note that when naïve T cells were co-cultured with DCs at a lower stimulator/responder ratio (1 : 100) there was no difference between pollen-DCs or iDCs (data not shown). In preliminary experiments we have found that inclusion of IL-2 (25 U/ml) during last 5 d of primary culture did not affect the polarization profile of T cells.

To further explore the cytokine production profile of T cells primed with pollen-stimulated DCs, these cells were stimulated with PMA and ionomycin and their produc-

tion of IFN- γ and IL-4 was examined at the single cell level by intracytoplasmic staining and FACS analysis (Fig. 5B). iDCs, pollen-primed DCs and LPS-primed DCs did not differ in their ability to induce low numbers of IL-4 producing T cells. Pollen-primed DCs induced more IFN- γ producing T cells than iDCs but less than LPS-primed DCs.

Collectively, the data indicate that pollen-primed DCs promote the development of naïve CD4⁺ T cells into effector cells with mixed profile of cytokine production.

The effect of pollen on DCs is contact-dependent

To assess the requirement of cell-grain contact in achieving aforementioned effects of pollen on DCs, transwell cultures were established. Pollen grains were confined to the upper well of a 0.45 μ m-porosity transwell chamber above a culture containing iDCs. Culture supernatants and cells were removed from the lower chamber after 48 h of co-culture and assayed for cytokine production and CD80, CD86 expression.

Culture supernatants revealed no difference in cytokine production between iDCs and DCs treated with pollen in transwell system (data not shown). There was no effect of pollen on CD80 and CD86 expression on DCs in transwell system, indicating the requirement for contact-dependent interaction (Fig. 6).

Discussion

A number of cell types including epithelial cells and antigen-presenting cells such as DCs and macrophages, all of which are capable of interacting with inhaled allergens, are present in the lumen of the respiratory tract. Here we demonstrate for the first time the biological activity of intact grains of pollen *in vitro* on human dendritic cells. Our results suggest that pollen grains exert an adjuvant effect and, thereby, promote the induction of an immune response to a biologically inert protein carried by the pollen particle in allergic and nonallergic individuals. Although surprising, our results are in line with the recent observations on human neutrophils and eosinophils demonstrating that grains of pollen influence their effector functions by inducing their directed migration and secretion of granular proteins in an allergen-independent manner (14, 15).

In our hands, exposure to pollen grains mediated on immature DCs a distinct maturation program characterized by a strong up-regulation of CD54, CD80, CD83, CD86, HLA-DR and the chemokine receptor CCR7 contrasting with low levels of CD40. This functional modulation of DCs was distinct from the classical DC activation triggered by LPS and, most importantly, it was not mediated by purified allergen from pollen AgE, that did not affect the expression of co-stimulatory molecules on immature DCs and cytokine production by DCs (data

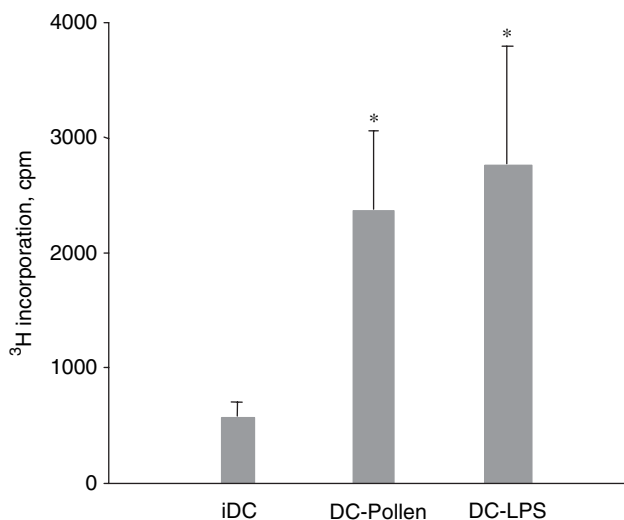


Figure 4. T cell priming activity of pollen-treated DCs. Naïve cord blood-derived CD4⁺ T cells were co-cultured for 7 days with DCs primed with pollen or LPS. 1 μ Ci/well ³H-thymidine was added last 6 h of culture. *Significantly different compared with cells co-cultured with unprimed DCs (iDC). Mean $\pm$ SEM of nine independent experiments.

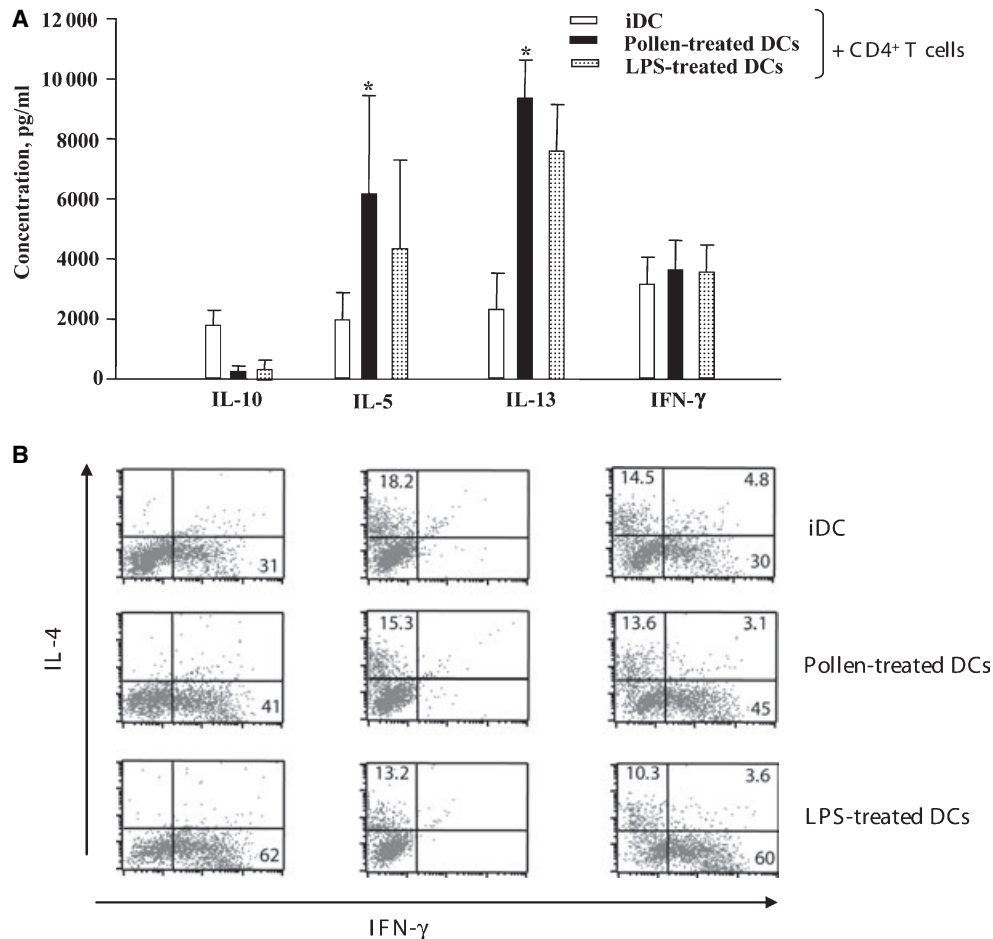


Figure 5. Polarization of naïve $CD4^{+}$ T cells by pollen-primed DCs. (A) Pollen-treated DCs promote differentiation of naïve T cells into high IL-5 and IL-13 producers. Cord blood-derived naïve $CD4^{+}$ T cells were co-cultured for 7 days with iDCs, pollen- or LPS-treated DCs. Cells were re-stimulated with anti-CD3 (100 ng/ml) immobilized on mitomycin C-treated CD32/B7.1 L cells (10^5 cells/ml) for 48 h. *Significantly different compared with cells co-cultured with unprimed DCs (iDC). Mean $\pm$ SEM of eight experiments. (B) Intracytoplasmic staining for cytokine production by $CD4^{+}$ T cells co-cultured for 7 days with differentially treated DCs (iDCs, pollen- or LPS-treated DCs). Cells were stimulated with PMA/ionomycin for 6 h in the presence of monensin for the last 5 h. Percentage of positive cells is shown in each quadrant. Data are representative of three experiments.

not shown). Migration of CCR7-expressing DCs to the afferent lymphatics and into the T cell area is selectively promoted by the presence of PGE_2 that facilitates the coupling of CCR7 to its signal transduction modules (25). Recently it has been shown that pollen grains release an exudate containing 'LTB₄-like' and PGE_2 -like' substances upon contact with the aqueous phases (14, 26). The recruitment of T cells into cell cycle and the subsequent rate of division have been reported to be enhanced by co-stimulatory molecules such as B7.1 and B7.2 (27). Likewise, our data have demonstrated that pollen-primed DCs acquired enhanced T-cell stimulatory capacity.

Skewing of naïve T cells toward a Th1 or Th2 response is a crucial process in determining the ultimate outcome of the immune response, and this is affected by the microenvironment of antigen-presenting DCs, as well as by the modulation of T-cell-receptor-mediated activation

signals (28, 29). In our hands, T cells primed by DCs treated with grains of pollen showed increased Th2 cytokines production, such as IL-5 and IL-13. However, at the single-cell level the percentage of IFN- γ -producing cells was higher than that of IL-4-producing cells. Therefore, in our culture conditions pollen-primed DCs induced a mixed Th-cell polarization.

Our findings show that when DCs were exposed to grains of pollen, they did not produce or produce at very low levels cytokines IL-12, IL-10 and TNF- α . This is consistent with the profile of semi-mature DCs since the characteristics of these semi-mature but tolerogenic DCs are their expression of co-stimulatory molecules, but low or absent production of pro-inflammatory cytokines, in particular IL-12 (23). The finding that upon secondary stimulation pollen-primed DCs, in contrast to LPS-primed DCs, produced significant amounts of cytokines

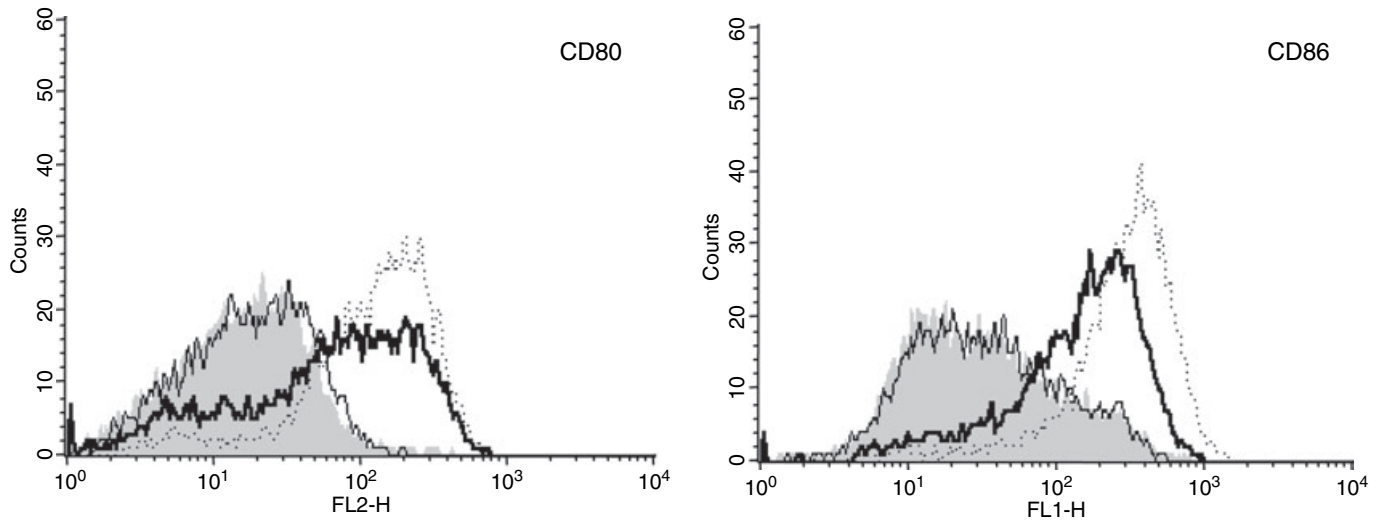


Figure 6. Effect of grains of pollen on DCs is contact-dependent. The expression of CD80 and CD86 on iDCs (filled histogram), DCs treated with pollen grains (thick line), pollen grains in Transwell system (thin line) or LPS (dotted line) was analyzed. Data are representative of three experiments.

and were not 'exhausted' indicates their semi-mature state as well (24). The conditions of activation and degree of maturation of the immature DCs are crucial for the signals that are delivered to the T cells. Activation of DCs without concomitant full DC maturation most of time leads to the development of regulatory T cells (30). However, we found no evidence for such regulatory T cell induction by pollen-primed DCs in our culture conditions (data not shown).

A further question related to present data was how intact grains of pollen would have an access to DCs in physiological conditions. Several scenarios may be envisaged. Firstly, study showing an Ag-specific T cell activation and proliferation occurring after intra-tracheal instillation of Ag-pulsed DCs was taken to indicate that these DCs may cross the epithelial tight-junction barrier and go from the airways to the lymph nodes (31, 32). Secondly, DCs penetrate the tight junctions between epithelial cells and send dendrites outside the epithelium allowing them to directly sample the small fragments of particles of pollen as it has been described for some bacteria (33). Intact hydrated pollen grains generate reactive oxygen species (ROS) due to intrinsic NAD(P)H oxidase in them (34). Thus, it is tempting to speculate that

these ROS could be implicated in the opening of the tight junction allowing the extrusion of dendrites. Finally, the particles (0.5–5 µm) released from grains of pollen were visualized inside of the cultured epithelial cells upon their exposure to pollen (34). This may represent another mechanism whereby pollen through the release of smaller particles gains an access to epithelial cells and DCs. This latter possibility may be attributed to the observation that the adjuvant effect of intact grains of pollen was contact-dependent (the particles did not pass through 0.45 µm Transwell system) and not related to the mediator release.

In summary, we provide evidence that pollen grains not only function as allergen carriers, but may have far more effects on human health by contributing to the manifestation or aggravation of allergic inflammation by virtue of their adjuvant effect on DCs.

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