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Pollen-Induced Oxidative Stress Influences Both Innate and Adaptive Immune Responses via Altering Dendritic Cell Functions

Aniko Csillag,* Istvan Boldogh,[†] Kitti Pazmandi,* Zoltan Magyarics,* Peter Gogolak,* Sanjiv Sur,[‡] Eva Rajnavolgyi,* and Attila Bacsı*

It has been demonstrated that pollen grains contain NAD(P)H oxidases that induce oxidative stress in the airways, and this oxidative insult is critical for the development of allergic inflammation in sensitized mice. On the basis of this observation, we have examined whether pollen grain exposure triggers oxidative stress in dendritic cells (DCs), altering their functions. To test this hypothesis, human monocyte-derived DCs were treated with ragweed pollen grains. Our findings show that exposure to pollen grains induces an increase in the intracellular levels of reactive oxygen species in DCs. Our data also indicate that besides the NAD(P)H oxidases, other component(s) of pollen grains contributes to this phenomenon. Elevated levels of intracellular reactive oxygen species triggered the production of IL-8 as well as proinflammatory cytokines, such as TNF- α and IL-6. Treatment with pollen grains initiated the maturation of DCs, strongly upregulated the membrane expression of CD80, CD86, CD83, and HLA-DR, and caused only a slight increase in the expression of CD40. The pollen-treated DCs induced the development of naive T lymphocytes toward effector T cells with a mixed profile of cytokine production. Antioxidant inhibited both the phenotypic and functional changes of DCs, underlining the importance of oxidative stress in these processes. Collectively, these data show that pollen exposure-induced oxidative stress may contribute to local innate immunity and participate in the initiation of adaptive immune responses to pollen Ags. *The Journal of Immunology*, 2010, 184: 000–000.

Dendritic cells (DCs) are characterized as the most effective APCs and conductors of immune defense by linking innate and adaptive immune responses. They form a cellular network at body sites continuously exposed to the environment, such as the skin, the gut, and the lung (1). Immature DCs are specialized for integrating signals from their environment, and while internalizing self- and foreign Ags, they use TLRs and other pattern recognition receptors to detect pathogen- or damage-associated molecules. Exogenous microbial products or signs of endogenous tissue damage induce the release of proinflammatory cytokines, chemokines, and antimicrobial peptides from DCs that initiate a rapid response to invading pathogens or harmful effects (2). In addition to their role in innate immunity, DCs also have the capacity to prime adaptive immune defense mechanisms. If DCs sense danger signals during Ag

capture, they launch their activation/maturation program that involves the downregulation of Ag internalization and migration to the draining lymph nodes to encounter and activate naive T lymphocytes (1). In the lymph nodes, mature DCs express elevated levels of costimulatory molecules (CD40, CD80, and CD86) and produce T cell-polarizing cytokines (1). The polarization of naive Th cells toward Th1, Th2, or regulatory T cells (Tregs) depends on Ag-derived and costimulatory signals as well as on the secreted cytokine profile provided by the interacting APCs.

Atopic allergy is considered as a Th2 cell-mediated inflammatory disease, because Th2-derived cytokines could promote mast cell development, Ig isotype switch to IgE, and activation of eosinophils (2). Although many details of the mechanism by which inhaled allergens induce the development of clinical symptoms in sensitized patients are relatively well understood, several unresolved questions still remain relating to the allergic sensitization process. It is unknown how harmless pollen-derived proteins can activate DCs, trigger their maturation, and consequently induce adaptive immune responses.

Recently, it has been shown that pollen grains and their allergenic extracts have a potent pro-oxidant activity, which induces profound oxidative stress in the lung or conjunctiva within minutes after exposure (3–5). Inhibition of this immediate oxidative insult significantly decreases the allergic inflammation in sensitized mice after allergen challenge (6). It has also been demonstrated that the pro-oxidant effect of pollen grains or extracts is due to the activity of NAD(P)H oxidases (3). Pollen NAD(P)H oxidases are partly homologous to the gp91^{phox} subunit of the mammalian phagocyte NADPH oxidases, and they have the same O₂^{•-} generating ability (7). Several lines of evidence suggest that reactive oxygen species (ROS) derived from NADPH oxidase activity are involved in the polarized growth of pollen tubes (8).

In this study, we demonstrate that oxidative stress induced by exposure to hydrated pollen grains may contribute to local innate immune responses by triggering proinflammatory cytokine production

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Abbreviations used in this paper: AU, arbitrary unit; DC, dendritic cell; DCF, dichlorofluorescein; DPI, diphenyleneiodonium; H₂DCF-DA, 2',7'-dihydro-dichlorofluorescein diacetate; IDC, untreated dendritic cells; LPS, LPS-treated DCs; Nrf2, NF-erythroid 2-related factor 2; PBN, *N*-tert-butyl- α phenylnitron; RFI, relative fluorescence intensity; ROS, reactive oxygen species; RWP, ragweed pollen grain; RWP^H, heat-treated ragweed pollen grain; Treg, regulatory T cell.

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of DCs. Moreover, elevated levels of intracellular ROS upon pollen exposure also affect the maturation/activation process of DCs; hence, they participate in the initiation of pollen Ag-dependent adaptive immune responses.

Materials and Methods

Generation of DCs

Leukocyte-enriched buffy coats were obtained from 10 healthy blood donors drawn at the Regional Blood Center of Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Debrecen, Hungary). Written informed consent was obtained from the donors prior blood donation, and their data were processed and stored according to the directives of the European Union. PBMCs were separated by a standard density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation with anti-CD14 microbeads according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). After separation on a VarioMACS magnet, 96–99% of the cells were CD14⁺ monocytes as measured by flow cytometry (data not shown). Monocytes were cultured in 12-well tissue culture plates at a density of 2×10^6 cells/ml in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium) and 100 ng/ml IL-4 (Peprotech EC, London, U.K.). On day 2, the same amounts of GM-CSF and IL-4 were added to the cell cultures. The individual DC cultures were characterized on day 5 by the expression of DC-SIGN/CD209, CD11c, HLA-DR, CD14, and CD1a. Immature DCs were found to be DC-SIGN/CD209⁺, CD11c⁺, HLA-DR⁺, and CD14^{low}, and the percentage of CD1a⁺ DCs varied among individuals (25–96%) (data not shown).

Treatments of DCs

On day 5 of the culture, immature DCs were exposed to 100 µg/ml common ragweed (*Ambrosia artemisiifolia*) pollen grains (RWPs; Greer Laboratory, Lenoir, NC), which were previously hydrated in AIM-V medium for 10 min. In preliminary experiments, different concentrations of RWPs (ranging from 5–400 µg/ml) were analyzed for their ability to increase the intracellular ROS levels in DCs, and a plateau was reached at 100 µg/ml concentration. Exposure to 100 µg/ml pollen suspension resulted in a ratio of 1 pollen grain to 100 cells. In control experiments, DCs were exposed to heat-treated (72°C for 30 min) ragweed pollen grains (RWP^Hs) (4). To investigate the effects of oxidative stress on DC function, cells were pretreated for 1 h with antioxidant (10 mM *N*-tert-butyl-α phenylnitron [PBN]; Sigma-Aldrich, St. Louis, MO) (9) before addition of pollen suspension. To analyze cytokine secretion of DCs, the cell culture supernatants were collected at 24 h after treatments and stored at –20°C until cytokine measurements.

Measurement of ROS

Untreated, 5-d-old immature DCs and DCs pretreated with PBN were loaded with 50 µM 2',7'-dihydro-dichlorofluorescein diacetate (H₂DCF-DA; Molecular Probes, Eugene, OR) at 37°C for 30 min. After removing excess probe, the cells were exposed to RWP, RWP^H, RWP with PBN, or RWP pretreated with diphenyleiiodonium (DPI) 100 µM (Sigma-Aldrich), respectively. Changes in fluorescence intensity were assessed in a Synergy HT micro plate reader (Bio-Tek Instruments, Winooski, VT) at 488-nm excitation and 530-nm emission.

Analysis of cell surface receptor expressions by flow cytometry

Phenotypic characterization of DCs was performed by flow cytometry using fluorochrome-conjugated Abs: anti-CD83-FITC, anti-CD86-PE, anti-HLA-DR-FITC (BD Pharmingen, San Diego, CA), anti-CD80-FITC, and anti-CD40-PE (Immunotech, Marseille, France). Isotype-matched control Abs were obtained from BD Pharmingen. Fluorescence intensities were measured by a FACSCalibur flow cytometer (BD Immunocytometry Systems, Franklin Lakes, NJ), and data were analyzed using WinMDI software (J. Trotter, The Scripps Research Institute, La Jolla, CA).

Proliferation of T cells

Naive CD4⁺ T cells were purified from PBMCs by negative selection using the naive CD4⁺ T cell isolation kit (Miltenyi Biotec). T cell preparations contained 96–98% CD4⁺ cells, of which 90–95% were CD45RA^{high} and

1.8–2.1% CD45RO⁺ as measured with flow cytometry (data not shown). DCs exposed to RWP in the presence or absence of PBN or to RWP^H were cocultured with allogeneic naive CD4⁺ T cells, which were previously labeled with 0.5 µM CFSE (Molecular Probes), for 4 d in the presence of 0.5 µg/ml purified anti-human CD3 (BD Pharmingen) at the ratio of 1:20. T cells stimulated with 10 µg/ml PHA (Sigma-Aldrich) were used as positive control. Fluorescence intensities were measured by a FACSCalibur flow cytometer, and data were analyzed by WinMDI software.

T cell activation by autologous DCs

To investigate the cytokine secretion profile of T lymphocytes primed with pollen-exposed DCs, monocytes as well as naive CD4⁺ T cells were isolated from buffy coats obtained from three ragweed allergic and three non-allergic blood donors out of the ragweed pollen season (February–June 2009). After receiving the buffy coat fractions of the blood samples, sensitization to ragweed pollen allergens was assessed via screening for total and specific IgE by means of ELISA (Adaltis Italia, Bologna, Italy). Sera containing <0.36 kU/L ragweed-specific IgE and a low level of total IgE (<20 IU/ml) were classified as “nonallergic,” and those with elevated ragweed-specific IgE levels (0.72–17.99 kU/L) were regarded as “ragweed allergic” samples. Monocytes and naive CD4⁺ T cells were isolated as described above. Pollen-treated and untreated DCs were washed and cocultured with autologous naive CD4⁺ T cells on 96-well tissue culture plates for 4 d at cell densities of 2×10^4 DCs/well and 2×10^5 T cells/well (at the ratio of 1:10) in AIM-V medium. After removing the T cells from the adherent DCs, they were reactivated for 24 h on plates coated with 5 µg/ml anti-CD3 mAb (BD Pharmingen). Supernatants of T cells were collected and used for cytokine measurements.

Cytokine measurements

A profile of cytokine release from DCs was determined by using ELISA. ELISA kits specific for IL-6, IL-8, IL-10, IL-12(p70), TNF-α, IL-1β, and IFN-γ were purchased from BD Pharmingen. Secreted cytokines by T cells were determined by cytometric bead array according to the manufacturer's instructions. The use of the Human Allergy Mediators Kit (BD Biosciences) allowed us the simultaneous measurement of the levels of IL-3, IL-4, IL-5, IL-7, IL-10, and GM-CSF in the samples. Fluorescence intensities were measured with a FACSCalibur flow cytometer, and the results were evaluated by the FCAP array software (BD Pharmingen). Secreted IFN-γ was determined from the supernatants of T cell cultures by using the human IFN-γ ELISA set (BD Pharmingen).

Characterization of IL-10-producing T cells

Anti-CD25-FITC (BD Pharmingen) Abs were used for the membrane staining of T cells. After a fixation/permeabilization step, the T lymphocytes were stained with Foxp3-PE (eBioscience, San Diego, CA) and IL-10-APC (Miltenyi Biotec) Abs. Intracellular Foxp3 and IL-10 staining was performed by using eBioscience reagents according to the manufacturer's protocol. To detect intracellular IL-10 cytokine production, monensin (GolgiStop; BD Biosciences) was added to the anti-CD3-restimulated autologous T lymphocytes during the last 6 h of the stimulation. Isotype-matched control Abs were obtained from BD Pharmingen. Fluorescence intensities were measured by a FACSCalibur flow cytometer, and data were analyzed by FlowJo software (Tree Star, Ashland, OR).

Statistics

One-way ANOVA followed by Bonferroni (equal variances assumed) or Dunnett T3 (unequal variances assumed) post hoc test was used for multiple comparisons. The Pearson's χ^2 test was applied to compare the distributions of the differently primed T cell populations. All analyses were performed by using SPSS Statistics software, version 17.0. Differences were considered to be statistically significant at $p < 0.05$.

Results

Pollen grains induce oxidative stress in monocyte-derived DCs

Airway DCs, predominantly located underneath the epithelial basement membrane, extend long interepithelial pseudopods toward the airway lumen (10); thus, they are able to come into direct contact with inhaled pollen grains. We used human monocyte-derived DCs to investigate the consequences of pollen exposure on DC function. Because it recently emerged that RWPs possess NAD (P)^H oxidase activity, which generates reactive oxygen radicals (3, 4), we presumed that exposure to pollen grains would increase the

intracellular level of ROS in cultured monocyte-derived DCs. To test this hypothesis, immature DCs were loaded with redox-sensitive $H_2DCF\text{-}DA$, and RWPs were added to the cell culture. Pollen exposure rapidly induced a 5.9 ± 2.1 -fold increase of intracellular dichlorofluorescein (DCF) fluorescence, which could be prevented by heat treatment of the pollen grains (Fig. 1). Pretreatment of RWPs with DPI, a NAD(P)H oxidase inhibitor, also significantly decreased the elevation of intracellular ROS levels (Fig. 1). To further confirm that ROS generated by pollen grains provoked increased DCF fluorescence in DCs, we applied PBN, one of the most successful spin-trapping agents used for identifying free radicals, such as the hydroxyl radical and the superoxide anion (11). The capacity of PBN molecules to neutralize free radicals provides functional activities similar to those of antioxidants (12). The presence of PBN in the cell culture medium did not significantly change the basal level of intracellular DCF fluorescence (data not shown); however, it attenuated the ragweed pollen-induced oxidative stress in DCs (Fig. 1). These data indicate that pollen exposure is able to induce oxidative stress in DCs, and this phenomenon could be inhibited by antioxidant as well as physical or chemical inactivation of pollen NAD(P)H oxidases.

Oxidative stress upregulates IL-8, TNF- α , and IL-6 synthesis by DCs upon pollen exposure

Previous studies have demonstrated that ROS either directly (13) or via oxidatively modified glycoproteins (14) are able to evoke the production of cytokines that are critical for triggering of innate immunity. To assess the potential effects of ROS generated by pollen grains on DCs, we measured IL-8 chemokine and proinflammatory cytokine secretion. At 24 h after administration of pollen grains, the levels of released IL-8 (7.4 ± 1.3 -fold increase), TNF- α (150.5 ± 60.1 -fold increase), and IL-6 (9.3 ± 2.6 -fold increase) were significantly higher in the supernatant of pollen-exposed DCs than in those of unstimulated cells (Fig. 2A–C). However, treatment of DCs with pollen grains did not induce the secretion of IL-1 β at any time points (data not shown). To determine whether the chemokine and cytokine release from DCs were induced by the oxidative insult, we treated the cells with pollen grains in the presence of PBN. The antioxidant decreased the amounts of IL-8, TNF- α , and IL-6 released by pollen-exposed DCs to the basal levels (Fig. 2A–C). Pretreatment with PBN alone did not affect the chemokine and cytokine release from DCs (data not shown). Surprisingly, the heat pretreatment of pollen grains, which eliminates the activity of the intrinsic pollen NAD(P)H oxidases (4), was not able to completely inhibit the mediator release (Fig. 2A–C). It has previously been reported that administration of LPS can also induce oxidative stress and trigger the secretion of various cytokines from monocyte-derived DCs (15).

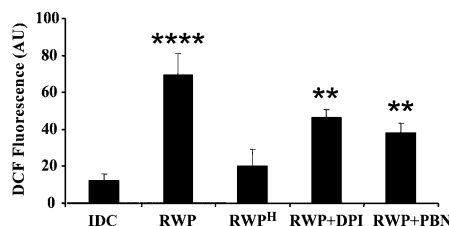


FIGURE 1. Exposure to RWPs increases the intracellular ROS levels in cultured monocyte-derived DCs. Cells were loaded with $H_2DCF\text{-}DA$ and, after removing excess probe, treated as indicated. Changes in DCF fluorescence intensity were detected by means of fluorimetry. Data are presented as means $\pm$ SEM of four independent experiments. The p values were calculated with one-way ANOVA followed by Dunnett T3 post hoc test. ** $p < 0.01$; **** $p < 0.0001$ versus IDC control. AU, arbitrary unit; IDC, untreated DCs; RWP, DCs exposed to RWPs; RWP^H, DCs exposed to heat-treated RWPs.

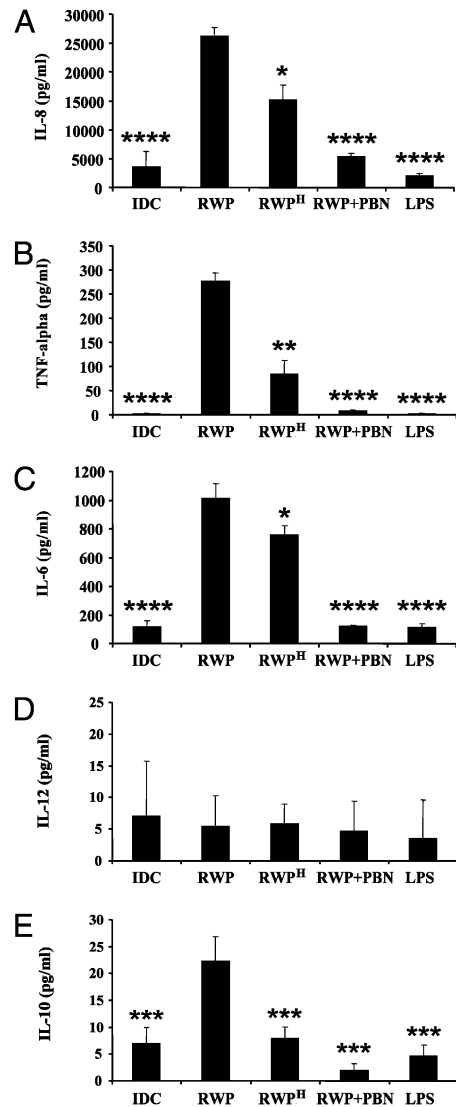


FIGURE 2. Effect of pollen exposure induced oxidative stress on the chemokine and cytokine-producing capacity of DCs. Levels of IL-8 (A), TNF- α (B), IL-6 (C), IL-12(p70) (D), and IL-10 (E) in the culture supernatants of pollen-treated DCs were determined 24 h after the exposure by means of ELISA. LPS contamination of RWP sample was determined, and the equivalent amount of LPS from *E. coli* (16 pg/ml) was used as control. Data are presented as means $\pm$ SEM of four to five independent experiments. The p values were calculated with one-way ANOVA followed by Bonferroni post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ versus pollen-treated DCs. IDC, untreated DCs; LPS, LPS-treated DCs; RWP, DCs exposed to RWPs; RWP^H, DCs exposed to heat-treated RWPs.

To exclude the possibility that pollen-induced DC activation might be due to LPS contamination of the RWPs, we determined the LPS content of our pollen samples. The analysis of ragweed pollen samples was performed by using the *Limulus* amoebocyte lysate test and revealed negligible quantities of LPS activity. In our further experiments, we used the equivalent amount of LPS from *Escherichia coli* (16 pg/ml) as a control. Treatment of immature DCs with this amount of LPS could not induce the release of IL-6, IL-8, or TNF- α from the cells (Fig. 2A–C).

Pollen exposure-triggered oxidative stress contributes to the phenotypic maturation of DCs

Phenotypic and functional changes of DCs during delivery of Ag to local lymphoid tissues allow them to prime naive T lymphocytes and initiate adaptive immune responses. To investigate whether ROS

produced by pollen grains contribute to the phenotypic maturation process, immature monocyte-derived DCs were incubated with RWPs for 24 h in the presence or absence of PBN. For comparison, immature DCs were also treated with LPS (16 pg/ml). The expression of costimulatory molecules (CD40, CD80, and CD86) CD83, a specific maturation marker, and the Ag-presenting molecule HLA-DR was analyzed by flow cytometry. Treatment of immature DCs with pollen grains resulted in a slight increase in the expression of CD40 (relative fluorescence intensity [RFI] increased from 20.80 to 25.83), whereas it markedly upregulated the expression of CD80 (RFI from 0.78 to 1.20), CD86 (RFI from 2.04 to 7.63), CD83 (RFI from 0.09 to 0.37 and frequency from 9.16% to 21.41%), and HLA-DR (RFI from 27.65 to 63.38) (Fig. 3). Heat pretreatment of pollen grains decreased, although not completely abolished, the ability of pollen grains to enhance the expression of activation and maturation markers on DCs (Fig. 3). The presence of PBN prevented the phenotypic shift of maturation triggered by pollen administration (Fig. 3); however, pretreatment with PBN alone did not modify the phenotypic characteristics of DCs (data not shown). The low con-

centration of LPS was not efficient to upregulate the costimulatory and maturation markers on the surface of DCs; thus, they remained at an immature state (Fig. 3).

Taken together, these results demonstrate that oxidative stress induced by pollen exposure is able to trigger enhanced synthesis of inflammatory mediators as well as phenotypic activation and maturation of DCs. Our observations indicate that besides the NAD (P)H oxidases, other component(s) of pollen grains also contribute to the increased ROS levels in DCs.

Pollen-induced oxidative stress alters the allostimulatory capacity of DCs

DCs are considered as the most potent APCs; therefore, we next studied the allostimulatory capacity of pollen-primed DCs. The responsiveness of CFSE-labeled naive CD4⁺ T lymphocytes to alloantigens presented by monocyte-derived DCs was analyzed by flow cytometry after 4 d of stimulation. The pollen-treated DCs exhibited a strong capacity to induce T lymphocyte proliferation. The cultures containing pollen-primed DCs had higher proportion

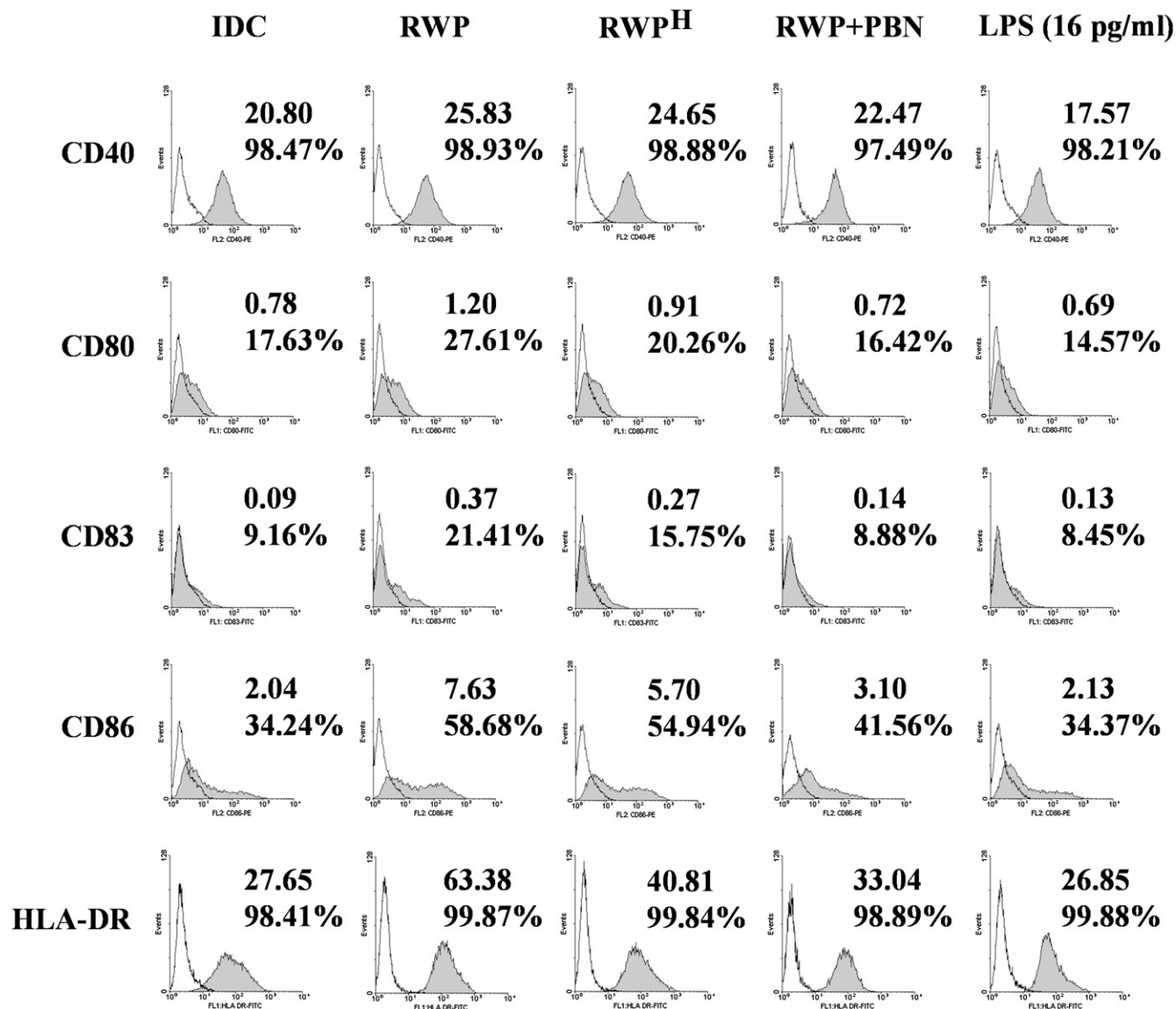


FIGURE 3. Pollen exposure-induced oxidative stress contributes to phenotypic maturation of DCs. Immature DCs were treated with RWP for 24 h, and the expression of HLA-DR, costimulatory molecules, or maturation marker was analyzed by flow cytometry. Unfilled histograms indicate isotype controls. Numbers indicate the RFI (upper) and the percentage of positive cells (lower). Results are representative of six independent experiments. IDC, untreated DCs; RWP, DCs exposed to RWPs; RWP^H, DCs exposed to heat-treated RWPs; LPS, LPS-treated DCs.

of dividing T cells as compared with those with immature DCs (80.8 versus 56.6%) (Fig. 4). To investigate whether pollen exposure-induced oxidative stress affects the allostimulation, heat-treated RWPs and PBN were applied. Exposure to heat-treated pollen grains or presence of PBN (71.6 and 60.5%, respectively) (Fig. 4) decreased the allostimulatory capacity of DCs. Pretreatment with PBN alone did not alter DCs' capacity to induce T cell proliferation (data not shown). Stimulation of DCs with LPS (16 pg/ml) resulted in a moderated proliferative response of allogeneic T cells (59.8%) (Fig. 4). As positive control, naive CD4⁺ T lymphocytes were labeled with CFSE and then assessed for their ability to proliferate when stimulated with the polyclonal T cell mitogen PHA for 4 d. Under the applied CFSE-labeling conditions, 78.6% of cells divided in response to PHA. These results provide evidence that the allostimulatory capacity of pollen-treated DCs depends, at least partly, on oxidative stress induced by pollen exposure.

Pollen-derived ROS change the T cell-polarizing capacity of DCs

DCs are responsible for directing different types of T cell responses, and the cytokine milieu around the interacting DCs and T cells apparently determines these processes. Next, we examined the IL-12 and IL-10 production of DCs at 24 h after pollen administration, because these cytokines play a pivotal role in the Th cell-polarizing activity of DCs. Immature DCs secreted very low amounts of IL-12 (p70), and administration of pollen grains in the presence or absence of antioxidant (PBN) or treatment with heat-inactivated pollen grains had no significant effect on basal IL-12 release (Fig. 2D). Pollen-treated DCs produced 22.2 ± 4.5 pg/ml IL-10, and this level of the cytokine was significantly higher than that released by untreated DCs (Fig. 2E). Exposure to heat-pretreated pollen grains did not enhance the IL-10 production of DCs. When PBN was added to the cell cultures, it notably inhibited the release of IL-10 from pollen-exposed DCs (Fig. 2E). In control experiments, IL-10 production of DCs was not affected by treatment with LPS (16 pg/ml).

To examine the role of pollen NAD(P)H oxidases in the T cell-polarizing capacity of DCs, the cytokine secretion profile of T lymphocytes isolated from peripheral blood of ragweed allergic and nonallergic subjects was analyzed after priming with pollen-exposed autologous DCs. Cytometric bead array was used for the measurement of the levels of IL-3, IL-4, IL-5, IL-7, IL-10, and GM-CSF. The amount of IFN- γ in the supernatants of the cell cultures was analyzed by ELISA. We were not able to detect the release of IL-7 from T cells in our experimental settings (data not shown). T lymphocytes from ragweed allergic subjects released significantly more IL-3 after priming with immature autologous DCs than those isolated from nonallergic subjects (37 ± 7.8 versus 12.3 ± 4.1 pg/ml) (Fig. 5A). T cells primed with pollen-stimulated autologous DCs produced higher amounts of Th2 cytokines, IL-4 and IL-5, than those primed with immature autologous DCs (Fig. 5B, 5C). The level of GM-CSF, an indicator of T cell differentiation regardless of Th1/Th2 commitment, was also higher in the supernatant of T cells primed with pollen-treated autologous DCs as compared with cells cocultured with untreated autologous DCs (Fig. 5D). However, no significant differences between the amounts of secreted IL-4, IL-5, or GM-CSF by T cells of different origin were found. Heat pretreatment of pollen grains decreased the capacity of pollen-exposed autologous DCs to induce cytokine release from T cells. The only exception to this observation was IL-10, because its production was significantly higher in the supernatant of T lymphocytes isolated from ragweed allergic persons and cocultured with heat-inactivated pollen-exposed autologous DCs (66.9 ± 15 versus 46.6 ± 9.6 pg/ml) (Fig. 5E). The same priming method did not stimulate increased release of IL-10 from T cells of nonallergic donors (Fig. 5E). T cells from nonallergic subjects produced 19.5-fold higher amount of IFN- γ after priming with pollen-treated autologous DCs, compared with those from allergic donors (7771 ± 2681 versus 399 ± 149 pg/ml) (Fig. 5F). Because it has previously been reported that antioxidants generate Tregs by inhibition of endogenous oxidative

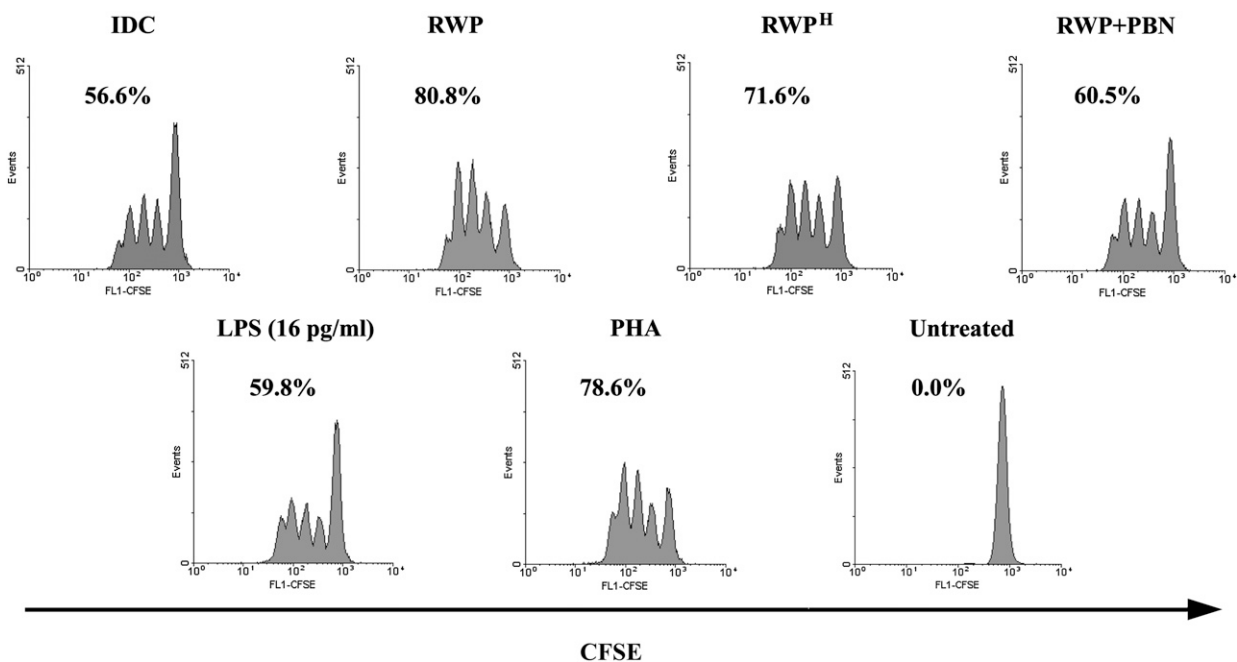


FIGURE 4. Increase in the intracellular ROS levels upon pollen exposure alters the T cell-priming capacity of DCs. Pollen-treated DCs were cocultured with CFSE-labeled allogeneic naive CD4⁺ T cells for 4 d, and then fluorescence intensities were measured by flow cytometry. Numbers indicate the proportion of dividing T cells. Results are representative of four independent experiments. IDC, untreated DCs; LPS, LPS-treated DCs; RWP, DCs exposed to RWPs; RWP^H, DCs exposed to heat-treated RWPs.

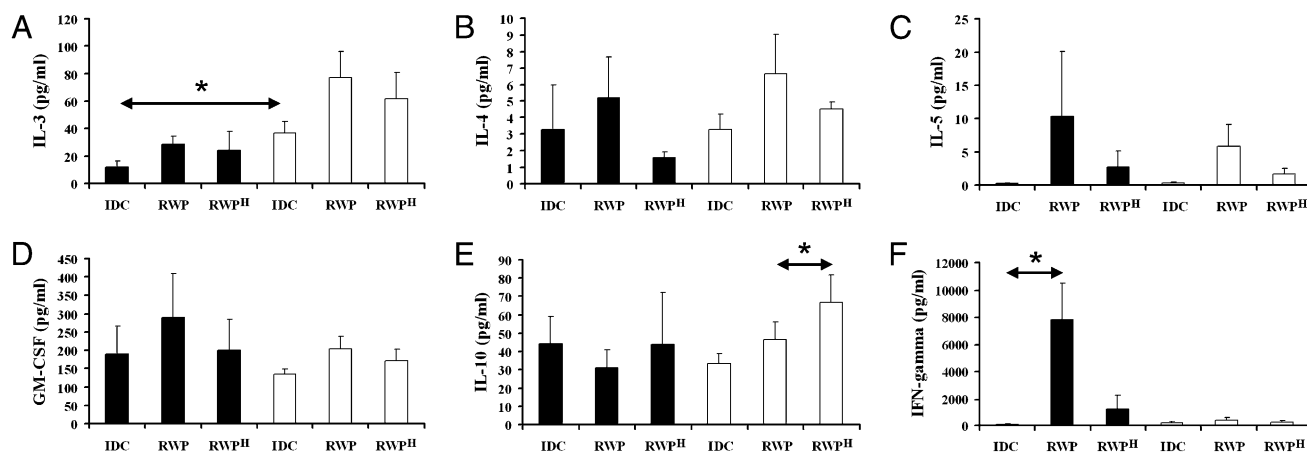


FIGURE 5. Pollen-induced oxidative stress influences the T cell-polarizing capacity of DCs. To investigate the cytokine secretion profile of T lymphocytes primed with pollen-exposed DCs, monocytes as well as naive CD4⁺ T cells were isolated from buffy coats obtained from three ragweed allergic (□) and three nonallergic blood donors (■). Pollen-exposed DCs were cocultured with autologous naive CD4⁺ T cells for 4 d, and T cells were then harvested and reactivated for 24 h. Supernatants of T cells were collected, and the levels of IL-3 (A), IL-4 (B), IL-5 (C), GM-CSF (D), and IL-10 (E) were determined by cytometric bead array, whereas concentrations of IFN-γ (F) were measured by means of ELISA. Data are presented as means ± SEM of three independent experiments. The *p* values were calculated with one-way ANOVA followed by Bonferroni post hoc test. **p* < 0.05. IDC, untreated DCs; RWP, DCs exposed to RWPs; RWP^H, DCs exposed to heat-treated RWPs.

pathways in human DCs (16), we did not use PBN treatment as control in these series of experiments.

To identify IL-10-producing T lymphocyte subpopulation(s) from ragweed allergic donors, first, the presence of CD25⁺Foxp3⁺ T cells was tested in the T cell cultures before and after priming with autologous DCs. Flow cytometric analysis of cells stained for CD4, CD25, and intracellular expression of Foxp3 showed that isolated naive T cells are contaminated by 0.85 ± 0.03% CD4⁺CD25⁺Foxp3⁺ T cells (data not shown). Priming T cells with immature DCs increased the proportion of CD25⁺Foxp3⁺ T cells up to 2.38 ± 0.42%.

After stimulation with pollen-exposed DCs, the ratio of this T cell population was 2.73 ± 0.27% (*n* = 3; *p* < 0.001 versus priming with immature DCs), whereas priming with heat-inactivated pollen-treated DCs further increased the rate of CD25⁺Foxp3⁺ T cells up to 3.61 ± 0.03% (*n* = 3; *p* < 0.001 versus priming with pollen-exposed DCs) (Fig. 6A). Simultaneous staining for CD25, intracellular IL-10, and Foxp3 identified a low ratio of IL-10-producing cells (0.28 ± 0.04%) (Fig. 6B, 6C). Priming with pollen-treated DCs did not change the ratio of IL-10⁺ T cells as compared with the untreated ones (0.27 ± 0.07%; *n* = 3; *p* = 0.417/NS). However, stimulation with

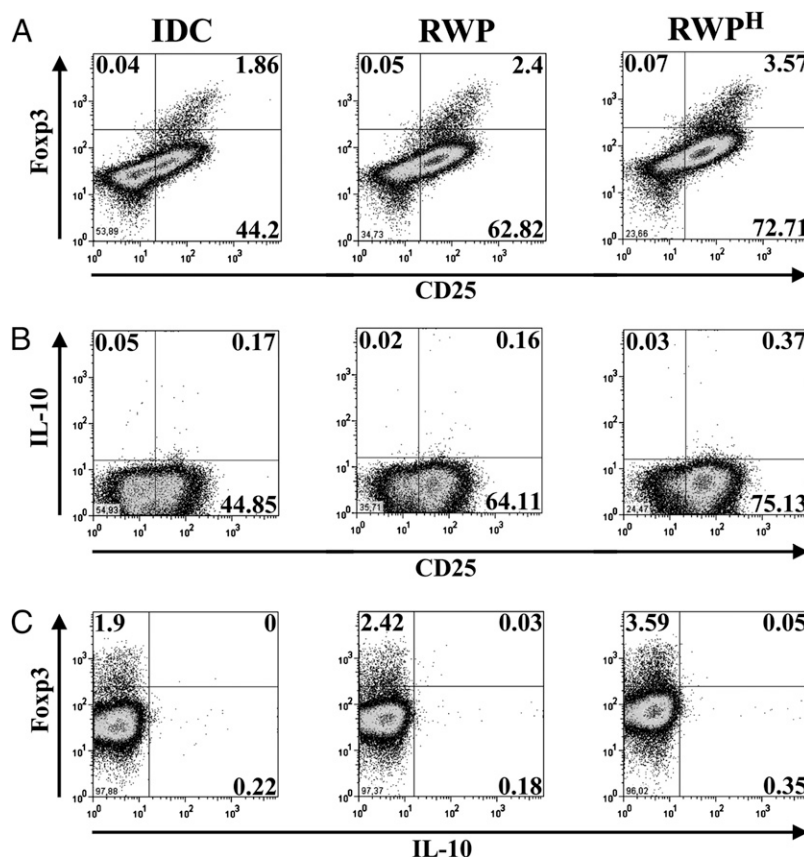


FIGURE 6. Characterization of IL-10-producing autologous T lymphocytes after coculturing with pollen-exposed DCs. The intracellular IL-10 and Foxp3 staining was performed after anti-CD3 reactivation of the DC-primed T cells from ragweed allergic subjects adding monensin in the last 6 h of stimulation. The density plots show staining for CD25-FITC and Foxp3-PE (A), CD25-FITC and IL-10-APC (B), as well as IL-10-APC and Foxp3-PE (C). The quadrant statistics were based on comparison of fluorescence intensities of isotype controls and specific Abs. Results are representative of three independent experiments. IDC, untreated DCs; RWP, DCs exposed to RWPs; RWP^H, DCs exposed to heat-treated RWPs.

heat-inactivated pollen-exposed DCs led to a 2.3-fold increase in the proportion of IL-10⁺ T cells ($0.625 \pm 0.18\%$; $n = 3$; $p < 0.001$ versus priming with pollen-exposed DCs) (Fig 6B, 6C). Results from this cytometric analysis indicate that CD25⁺/Foxp3⁺ T cells are the main source of IL-10 in the DC-primed T lymphocyte population (Fig. 6B, 6C). These data suggest that pollen-treated DCs are able to induce the differentiation of naive T cells toward effector T cells with a mixed profile of cytokine production, and heat inactivation of pollen NAD(P)H oxidases before DC treatment decreases the T cell-priming ability of DCs.

Discussion

RWP is one of the most important sources of the aeroallergens in many countries because it is responsible for the majority and most severe cases of seasonal rhinitis, conjunctivitis, and allergic asthma. The molecular analysis of ragweed pollen revealed Amb a 1, a pectate lyase enzyme, to be the predominant allergen, because >90% of the ragweed-sensitive subjects have Abs against this protein (17). Data from experimental animal models of allergic inflammation indicate that Amb a 1 requires priming in combination with adjuvants to overcome tolerogenic mechanisms that prevent allergic responses to inhaled Ags (18). Indeed, several lines of evidence suggest that pollen grains are not only carriers of allergenic proteins but also act as an adjuvant in the sensitization phase of the allergic reactions (19, 20). Hydrated pollen grains release serine and cysteine proteases (21–23), and it has been shown that cysteine proteases can induce the maturation of DCs directly, even in the absence of microbial stimuli; furthermore, protease-pulsed DCs mediate the polarization of the immune response toward a Th2 profile (24). Furthermore, induction of tolerance could be aborted and the allergic airway response triggered by the addition of purified protease to an innocuous Ag (25). In addition, it has been found that aqueous pollen extracts contain bioactive lipids that modulate both murine and human DC function in a fashion that favors Th2 cell polarization (26–28).

In this study, we report that the oxidative stress induced by exposure to pollen grains is able to activate DCs; thus, it may exert an adjuvant effect. Our finding that pollen exposure induces oxidative stress in DCs is in line with recent *in vitro* and *in vivo* data showing that intrinsic pollen NAD(P)H oxidases increase the intracellular levels of ROS in epithelial cells (4, 5). Our observation that heat pretreatment, which eliminates pollen NAD(P)H oxidase activity (4), did not completely abolish the ability of pollen grains to trigger oxidative stress in DCs indicates the contribution of other pollen component(s) to this phenomenon. A previous study has reported that LPS (100 ng/ml), which is recognized by TLR4, induces ROS generation in human monocyte-derived DCs (15). It has also been demonstrated that complex glucan structures bearing an $\alpha(1\text{--}3)$ -fucosylated core as well as membrane lipid peroxidation products can either directly or indirectly activate the TLR4 signaling cascade (29–31). Because RWPs contain $\alpha(1\text{--}3)$ -linked core fucosylated glucans (32) and pollen membranes are susceptible to enzymatic or free-radical-catalyzed peroxidation (33, 34), we suppose that these pollen grain components can induce oxidative stress in DCs via TLR4-mediated mechanism. However, future studies are needed to test this hypothesis.

Oxidative stress activates the NF- κ B and MAPK signaling pathways that are responsible for transcriptional activation of proinflammatory cytokine and chemokine genes in macrophages and DCs, respectively (13, 35, 36). Thus, increased production of IL-8, TNF- α , and IL-6 after pollen grain treatment, which could be reduced in the presence of antioxidant, corroborates the induction of oxidative stress in DCs. Our data showing that oxida-

tive stress induced by pollen exposure causes upregulation of costimulatory molecules and activation marker on the surface of DCs are in accordance with a previous study that has indicated that superoxide anions generated by the reaction of xanthine oxidase on xanthine induce phenotypic maturation of DCs by up-regulating CD80, CD83, and CD86 markers (37). In addition to the increased expression of costimulatory molecules, superoxide anion-treated DCs exhibit enhanced capacity to trigger T cell proliferation (37). There is ample evidence from a human study that oxidative stress can serve as a potent adjuvant in allergic sensitization. Atopic patients, who were intranasally exposed to a neoantigen, produced anti-neoantigen-specific IgE only when they were sensitized with the neoantigen in the presence of diesel exhaust particles possessing pro-oxidative properties (38).

From the perspective of allergic diseases, IL-12 production is a determining element of DC function, because low levels of IL-12 could favor Th2 differentiation (39). Our data indicate that pollen-exposed DCs produce IL-12 at a very low level. This confirms the previous observation that contact with pollen grains induces the development of semimature DCs (20). It has recently been reported that oxidative stress can activate the NF-erythroid 2-related factor 2 (Nrf2)-mediated signaling pathway that dominates over the TLR pathways, which promote IL-12 production (40). Thus, the Nrf2-dependent pathway may be responsible for the suppression of IL-12 generation in DCs (40). Furthermore, E₁-phytylprostanes, a class of PG-like lipid mediators of pollen grains, inhibit the LPS or CD40 ligation-induced production of IL-12 (26). Because E₁-phytylprostanes are formed nonenzymatically via reactive oxygen radicals from α -linolenic acid (26), ROS generated by pollen NAD(P)H oxidases may have a role in their synthesis. These findings suggest that pollen-derived reactive radicals could interfere with IL-12 generation of DCs at least in two different ways.

Previous data showed that oxidative stress can be induced in cultured epithelial cells through the direct contact with pollen grains (4). In our experiments, the expression of CD83 in the pollen-treated DC population also demonstrates that pollen exposure initiated the maturation program, however, only in a fraction of the cells. Note that in our cell culture system, the pollen grain-DC ratio was 1:100. DCs in the cell cultures were exposed to different levels of ROS, depending on their distance from the pollen grains. A recently proposed hierarchical oxidative stress model describes the relationship between the level of ROS and the level of cellular responses (35). Thus, lower levels of ROS leads to the translocation of Nrf2 to the nucleus, where this transcription factor initiates the expression of protective phase II enzymes, which exert antioxidant, detoxification, and anti-inflammatory effects (35). Higher level of oxidative stress activates the proinflammatory cascades as discussed above. We presume that during the analysis of the T cell-polarizing capacity of pollen-treated DCs, naive T cells could interact with DCs at different stages of their activation/maturation program that may explain why we could detect both Th1 and Th2 cytokines, as well as IL-10 in the supernatant of T cells primed with pollen-treated DCs. Our findings corroborate the earlier work, which reported that pollen-primed DCs promote the development of naive T lymphocytes into effector cells with a mixed profile of cytokine production (20). Although there are differences in the amount of the detected cytokines compared with previous observations (20), the discrepancies may be attributed to the serum-free medium used in our cell cultures or to the different methods applied for restimulation.

Our results, showing that after priming with pollen-treated DCs, CD4⁺ T cells of nonallergic individuals produce higher amounts of IFN- γ but release the same levels of Th2 cytokines (except IL-3) compared with those from ragweed allergic subjects, are in line with

previous observations showing a significantly higher percentage of IFN- γ -producing Th cells in normal control subjects than asthmatic patients, whereas there were no differences in the percentage of IL-4-producing Th cells (41). It has also been reported that IFN- γ production of effector T cells generated in vitro from naive precursors from patients with atopic dermatitis is decreased compared with that of healthy T cells (42). Although the mechanism of this phenomenon is not well understood, in a recent study, the IFN- γ gene polymorphism at position +874 has been linked to the intrinsic defect in the production of IFN- γ by Th1 cells in atopic individuals (43).

Although priming with pollen-exposed DCs increased the proportion of CD25⁺Foxp3⁺ T cells and stimulation with heat-inactivated pollen-treated DCs further enhanced the percentage of this T cell population, we found that CD25⁺Foxp3⁺ T cells are those responsible for elevated IL-10 production. Foxp3 is stably expressed in CD4⁺CD25⁺ Tregs; however, its transient expression has also been reported in human-activated nonregulatory CD4⁺ T cells (44). On the basis of this observation, it seems that most of the Foxp3⁺ cells are made up of activated T cells developed after in vitro stimulation of naive CD4⁺CD25⁺ T lymphocytes. Because priming with heat-inactivated pollen-treated DCs induced significant increase in IL-10 production only in T cells from ragweed allergic individuals, we presume that activation of memory effector T cells lies behind this phenomenon. Our observation that the isolated naive T cell population still contains trace amounts of CD4⁺CD45RO⁺ memory cells (1.8–2.1%) supports this hypothesis. Our data, however, do not exclude the possibility that naive T cells from atopic subjects possess elevated intrinsic ability to differentiate into Tr1 regulatory cells (CD4⁺CD25⁺FOXP3⁺IL-10⁺) after priming with DCs (45). The fact that DCs exposed to heat-treated pollen grains are more tolerogenic than pollen-stimulated ones further confirms the important role of pollen NAD(P)H oxidases in DC activation.

Our findings indicate that the deficiency of antioxidant defense mechanisms may increase the susceptibility to pollen-derived ROS or other environmental factors that promote allergic sensitization. This hypothesis is supported by the fact that genetic polymorphisms of GSTs, enzymes that metabolize ROS, have been identified as possible risk factors for the development of allergic airway responses. Individuals with GSTM1-null or the GSTP1-I105 wild-type genotypes respond with a more prominent increase in IgE and histamine levels after exposure to diesel exhaust particles plus allergen challenge than those carrying other genotypes (46) and exhibited a more intense nasal response to allergens inhaled with secondhand tobacco smoke as compared with clean air (47). Future studies performed with DCs from atopic and nonatopic patients are needed to analyze differences in their responses to oxidative stress.

In summary, we report that oxidative stress induced by hydrated pollen grains has dual impacts on DCs. It can trigger proinflammatory cytokine production from DCs contributing to local innate immunity and also act as an adjuvant factor in the initiation of adaptive immune responses against pollen Ags.

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Disclosures

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