SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Role of reactive oxygen species in the sensitization and elicitation phase of pollen-induced allergic reactions

by
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The Examination takes place at the Discussion Room of Building C, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 12 AM, 16th of September, 2014.

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 2 PM, 16th of September, 2014.

1. Introduction

The prevalence of allergic airway disorders, like asthma and allergic rhinitis, has dramatically increased in the last decades in industrialized countries. According to the World Health Organization (WHO), 300 million people suffered from asthma worldwide in 2005. These diseases characterized by significant negative effects on personal life quality, induce a considerable socio-economic impact due to health care utilization and treatment costs. As reported in 2009 approximately 50% of the Hungarian population was sensitized to a highly allergenic plant species, the common ragweed (*Ambrosia artemisiifolia*). These data indicate the highest prevalence of ragweed sensitization occurs in Hungary compared to other Central and Western European countries.

Allergic airway reactions mediated by IgE antibodies develop in two stages. The first one is an induction or sensitization phase which is followed by an elicitation phase.

1.1 Sensitization phase of airway allergy

In the initiation of allergic airway responses a specialized population of antigen presenting cells (APCs) called dendritic cells (DCs) play a fundamental role by bridging innate and adaptive immunity.

Several DC subsets have been indentified in specific anatomic locations in the airways with distinct functions. The trachea and large conducting airways contain a well-developed network of intraepithelial DCs. They extend their dendritic projections between epithelial cells, allowing them to sample airway lumen content, even in steady state conditions. Another airway DC subset resides in the submucosa of the conducting airways and in lung parenchyma. These cells exhibit an efficient capacity for priming and restimulating effector $CD4^+$ T cells in the lung. Lung plasmacytoid DCs (pDCs) in steady state represent only a minor population that can be found in the large conducting airways. These cells have been shown to contribute to the development of inhalation tolerance, i.e. after taking up inhaled harmless antigens they drive the formation of regulatory T cells (Tregs). The most important function of DCs is the capture and delivery of antigens to draining lymph nodes, where DCs can present the antigens to naïve T cells and induce T cell proliferation and differentiation.

To launch the activation/maturation program of DCs two signals are required. The first one principally originates from receptor-mediated antigen uptake, while the second signal is derived from sensing pathogen-associated molecular patterns (PAMPs) or danger signals (DAMPs) during antigen capture by DCs. Exogenous danger signals, which can be found in

the allergen itself, are capable to initiate the activation of DCs and this way promote the Th2 sensitization. Proteolytic activity as a general feature of major allergens has been proposed to be involved in the pathogenesis of allergies by enabling the passage of allergens through the epithelial barrier and modulating the functions of innate immune cells. Furthermore, pollens liberate bioactive lipids, the so-called pollen-associated lipid mediators and also contain adenosine which contributes to Th2 development. Additionally, pollen grains due to their NAD(P)H oxidase activity are able to generate reactive oxygen species (ROS) which provide a signal for the initiation of allergic inflammation.

After sensing and taking up antigens at mucosal surfaces by lung DCs, they migrate via the afferent lymphatics to the draining lymph nodes where present the processed antigens to T cells and initiate cognate T cell responses. This process is highly dependent on DC-derived signals, including a particular density of peptide-MHC class II complexes (signal 1), costimulatory cell surface molecules (signal 2) and soluble cytokines and chemokines (signal 3). Allergen activated Th2 cells produce copious amounts of IL-4, IL-5 and IL-13 leading to the accumulation of mast cells and eosinophils and switching to IgE isotype that occurs in B cells. IgE produced by B cells is released into the blood and quickly binds to high-affinity Fc receptors for IgE (FceR) I on the surface of mast cells and peripheral blood basophils resulting to become „armed" or sensitized.

1.2 Elicitation phase of allergic inflammation

Re-exposure to the allergen leads to crosslinking of IgE-binding FcεRI receptors on the surface of the mast cells. Following aggregation of FcεRI a complex intracellular signaling process is triggered that results in the secretion of various biologically active products. Mediators, such as those stored in preformed cytoplasmic granules, including biogenic amines, serine proteases and proteoglycans are released within minutes after antigen exposure. Similarly, the newly formed lipid-derived mediators, leukotriene B_4 (LTB_4), LTC_4, certain cytokines and growth factors that can be associated with the granules also produced by activated mast cells. The release of these mediators contributes to early phase reaction-associated symptoms such as increased vascular permeability, contraction of bronchial smooth muscle and elevated mucus secretion resulting in acute airflow obstruction and wheezing. In addition to FcεRI-mediated signals, alternative mechanisms have been shown to induce mediator release in mast cells. During allergic inflammatory reactions mast cells are exposed to ROS derived from various cell types and from the pollen itself. Recently it has been demonstrated that ragweed pollen extract activates RBL-2H3 cells (basophilic leukemia cell line) and induces the release of biogenic amines in an IgE-independent manner.

1.3 Oxidative stress in the airways

Allergic airway inflammation triggered by inhaled allergens is closely associated with oxidative stress that is defined by the excess of ROS which evolving by the unbalanced antioxidant and ROS scavenging mechanisms. In the airways both endogenous and exogenous sources of ROS play significant role in the development of oxidative stress.

Inflammatory cells such as macrophages, neutrophils and eosinophils are considered the primary source of endogenous ROS in the airways. These activated inflammatory cells can generate superoxide anions through NADPH oxidase pathway, cytosolic xanthine oxidase system and the mitochondrial respiratory chain. ROS production by neutrophils correlates with the severity of airway inflammation. Airway macrophages in patients who have asthma produce more superoxide anions than those of control subjects. Additionally, several studies have demonstrated that environmental factors such as ozone, diesel exhaust, cigarette smoke also contribute to the formation of ROS in the lungs and the augmentation of disease symptoms.

The oxidizing effect of ROS can be countered by reducing agents called antioxidants which can be classified according to their nature as enzymatic or non-enzymatic antioxidants. In the lungs, the main enzymatic reducers are superoxide dismutase (SOD), catalase (CAT), gluthatione peroxidase (GSH-Px) and thioredoxin (TRx). Among the non-enzymatic reducers we can find mucin, urate, gluthatione (GSH), ascorbate, ceruloplasmin, transferrin, vitamin E, ferritin and small molecules such as bilirubin.

In the last 10 years a new element was added to the big picture describing the relationship between oxidative stress and the initiation of allergic inflammation induced by airborne allergens. It turned out that pollen grain extracts of different plant species, among them numerous highly allergenic weed (including ragweed pollen), tree and grass pollens, have redox activity due to their intrinsic NAD(P)H oxidase activity. Hydrated pollen grains and subpollen particles (SPPs) released by them also contain NAD(P)H oxidases and their pro-oxidant activity plays an important role in the initiation of allergic inflammation. It was reported that ROS generated by pollen NAD(P)H oxidases induce oxidative stress in the lungs and conjunctiva of sensitized mice within minutes after exposure. This first innate signal recruits neutrophils into the lungs independent of the adaptive immune response.

1.4 Static magnetic field

Several studies have shown that magnetic fields influence a large variety of cellular functions, however, the mechanisms of interactions between static magnetic field (SMF) and living cells remain unclear. One possible mechanism can be the direct interaction of SMF

with free radicals affected by their membrane processes and related with the physiological functions. Another possible mechanism might be related to influencing antioxidant/oxidant status of the organism. It is assumed that SMF can change the lifetime of radical pairs. SMF can also influence the spin of electrons in free radicals, which may lead to changes in chemical reaction kinetics and possibly alter cellular function.

Many papers demonstrate a positive effect of SMF by decreasing oxidative stress index and lowering oxidative stress markers. However, these findings are in contrary with others' which showed that SMF stabilizes free radicals, thus increasing their overall concentration and dispersion within the cell resulting in oxidative injury. These controversial or inconsistent effects of SMF reported so far are particularly due to the diverse reactions of different cell types and living organisms and various intensity ranges of SMF. To investigate the effect of SMF on allergic airway inflammation induced by ROS producing ragweed pollen extract (RWPE) we used a device formerly developed by János László and his colleagues. This research group found that whole body exposure of mice to inhomogeneous SMF achieves a strong analgesic effect in the writhing tests and reduces inflammatory mechanical hyperalgesia. It was also demonstrated that SMF has an inhibitory effect on the release of pro-inflammatory cytokines from macrophages.

Based on these previous data, we investigated the effect of SMF-exposure on pollen-induced inflammatory airway disease in a mouse model. Currently, no published data are available about the effects of SMF-exposure on pollen-induced allergic inflammation.

2. Aim of the studies

- To examine whether pollen exposure triggers oxidative stress in human monocyte-derived DCs;

- To study whether oxidative stress induced by exposure to pollen grains contributes to innate immune responses by provoking proinflammatory cytokine production of DCs;

- To investigate whether pollen-derived ROS through altering DCs' functions participate in the initiation of pollen antigen-dependent adaptive immune responses;

- To check whether inhomogeneous SMF has any effect on pollen induced allergic airway inflammation in an *in vivo* model system;

- To investigate the mechanism of action by which SMF can influence allergic airway inflammation.

3. Material and Methods

3.1 Studies on pollen-exposed human monocyte-derived DCs

Isolation of monocytes and generation of DCs

Leukocyte-enriched buffy coats were obtained from 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). Human peripheral blood mononuclear cells (PBMCs) were separated from buffy coats by a standard density gradient with Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation with anti-CD14 conjugated microbeads (Miltenyi Biotec, Bergish Gladbach, Germany). To generate immature DCs (iDCs), cells were cultured in 12-well cell culture plates at a density of $2x10^6$ cells/ml in AIM-V medium (Invitrogen, Carlsbad, CA, USA) for 5 d. Medium was supplemented with 80 ng/ml GM-CSF (Gentaur Molecular Products, Brussels, Belgium) and 100 ng/ml IL-4 (Peprotech EC, London, UK) on days 0 and 2.

Treatments and characterization of DCs

On the fifth day of their differentiation iDCs were exposed to 100 µg/ml common ragweed (*Ambrosia artemisiifolia*) pollen grains (RWPs; Greer Laboratory, Lenoir, NC, USA), which were previously hydrated in AIM-V medium at room temperature for 10 min. In control experiments, DCs were exposed to heat-treated (72°C for 30 min) ragweed pollen grains (RWP^Hs). To study the effects of oxidative stress on DC function, antioxidant (10 mM N-tert-butyl-α phenylnitrone; PBN; Sigma-Aldrich, St. Louis, MO, USA) was added to the cell cultures 1 h prior to the pollen exposure. Supernatants were collected at 24 h after treatments and used for cytokine measurements.

Phenotypic characterization of DCs was performed after 24 h treatments by flow cytometry using fluorochrome-conjugated antibodies: anti-CD83-FITC, anti-CD86-PE, anti-HLA-DR-FITC (BD Pharmingen, San Diego, CA, USA), anti-CD80-FITC and anti-CD40-PE (Immunotech, Marseille, France). Isotype-matched control antibodies were obtained from BD Pharmingen. Fluorescence intensities were measured by FACSCalibur flow cytometer (BD

Biosciences Immunocytometry Systems, Franklin Lakes, NJ, USA) and data were analyzed using WinMDI software (Joseph Trotter, La Jolla, CA, USA).

Measurement of intracellular ROS levels in DCs

A redox-sensitive fluorescent dye, 2'-7'-dihydro-dichlorofluorescein diacetate (H_2DCF-DA, Molecular Probes, Eugene, OR, USA) was used to detect the levels of intracellular ROS upon RWP exposure. Untreated, 5-day-old immature DCs and PBN-pretreated DCs were loaded with 50 μM H_2DCF-DA at 37°C for 30 min. After removing excess probe, cells were exposed to RWP, RWP^H, RWP with PBN, or RWP pre-treated with NADPH oxidase inhibitor (diphenyleneiodonium, DPI), respectively. Changes in fluorescence intensity were assessed in a Synergy HT micro plate reader (Bio-Tek Instruments, Winooski, VT, USA) at 488 nm excitation and 530 nm emission.

T cell proliferation assay

Naïve $CD4^+$ T cells were purified from PBMCs by negative selection using the naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec). DCs exposed to RWP in the presence or absence of PBN or to RWP^H were cocultured with allogeneic naïve $CD4^+$ T cells, which were previously labeled with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes), for 4 days 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 phytohemagglutinin (PHA, Sigma-Aldrich) were used as positive control. Fluorescence intensities were measured by FACSCalibur flow cytometer and data were analyzed by WinMDI software.

T cell activation by autologous DCs

Naïve $CD4^+$ T cells were isolated from buffy coats obtained from 3 ragweed allergic and 3 non-allergic blood donors. Pollen-treated and untreated DCs were washed and cocultured with autologous naïve $CD4^+$ T cells on 96-well cell culture plates for 4 d at cell densities of 2×10^4 DCs/well and 2×10^5 T cells/well 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

Set of cytokines secreted by DCs was determined by using ELISA kits specific for human IL-6, IL-8, IL-10, IL-12(p70) and TNF-α (all from BD Pharmingen). To measure cytokines

secreted by T cells we used cytometric bead array according to the manufacturer's instructions. The use of 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 FACSCalibur 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 human IFN-γ ELISA set (BD Pharmingen).

Characterization of IL-10 producing T cells

After coculture with DCs, autologous T cells were reactivated for 24 h as described above. During the last 6 h of the stimulation, monensin (GolgiStop, BD Biosciences) was added to the anti-CD3-restimulated T lymphocytes in order to inhibit IL-10 cytokine release from the cells. For cell surface labeling of regulatory T cells we used anti-CD25-FITC (BD Pharmingen) antibodies. After a fixation/permeabilization step the T lymphocytes were stained with Foxp3-PE (eBioscience, San Diego, CA) and IL-10-APC (Miltenyi Biotec) antibodies. Isotype-matched control antibodies were obtained from BD Pharmigen. Fluorescence intensities were measured by FACSCalibur flow cytometer and data were analyzed by FlowJo software (Treestar, Ashland, OR, USA).

3.2 Studies on the effects of inhomogeneous SMF on allergic airway inflammation

Inhomogeneous SMF exposure system

The inhomogeneous SMF was generated with an exposure device consisted of 2 ferrous matrices containing 10x10 mm cylindrical neodymium iron boron (NdFeB) N35 grade magnets (B_r=1.20 T). The individual magnets in both matrices were placed next to each other with alternating polarity. Magnets facing each other in the 2 matrices were oriented with opposite polarity. The matrices were fixed in a holder in which the matrices were separated from each other with a distance of 50 mm. This arrangement allowed us to insert a 140x100x46 mm Plexiglas animal cage with ventilation holes on the front and back sides or 6-well cell culture plates into the space (exposure chamber) that separated the 2 matrices.

In order to test 2 different vertical magnetic induction values and corresponding lateral gradients simultaneously in a single exposure chamber, we planned our *in vitro* experiments with 2 layers. Two 6-well cell culture plates were stacked on top of each other. The SMF at the bottom of the lower culture plate in the stack was denoted by "lower" SMF, the top of the stack was the "upper" SMF. The height of a 6-well cell culture plate (12 mm) defined the

distance between the layers. Therefore the actual distance from the magnetic surface was 3 mm for the lower SMF and 15 mm for the upper SMF.

Typical peak-to-peak vertical magnetic induction values along the axis of a magnet in the isocenter of the generator in the *in vivo* and *in vitro* experiments were 389.46±0.1 and 2.97±0.1 mT, whereas the lateral gradient values between the 2 neighbouring local extremes were 39.25 and 0.22 T/m at 3 and 15 mm from the surfaces of matrices, respectively. Values of the SMF used in the skin prick test were: 192.28±0.1 mT by 18.89 T/m lateral gradient at 3 mm from the magnet.

Animals, sensitization, challenge and SMF exposure

Female 8-week-old Balb/c mice (Charles River, Wilmington, MA, USA) were used for these studies. For SMF-exposure 3 animals were put into the perforated cage at a time, then the cage with the animals was inserted into the exposure chamber of the magnetic device for 30 or 60 min. Sham-exposure was carried out by placing the 3 animals in identical cages without inserting the cage in the exposure chamber. All animals were sensitized with 2 intraperitoneal administrations (on days 0 and 4) of 150 µg/injection endotoxin-free ragweed pollen extract (RWPE, Greer Laboratories), combined in a 3:1 ratio with alum adjuvant (Pierce Laboratories, Rockford, IL, USA). On day 11, mice were challenged intranasally with 100 µg RWPE or PBS (PAA Laboratories, Pasching, Austria). On day 14, mice were euthanized and allergic inflammation was evaluated. All animal study protocols were approved by the Animal Care and Protection Committee at the University of Debrecen.

Evaluation of allergic inflammation

Inflammatory cell infiltration into the airways was assessed by the analysis of the bronchoalveolar lavage fluid (BALF) at 72 h after allergen challenge. Total cell counts in the BALF were determined from an aliquot of the cell suspension. Eosinophils, neutrophils, lymphocytes, and macrophages on Wright-Giemsa-stained cyto-centrifuge preparations were enumerated by counting at least 400 cells. To investigate lung histology, coronal sections of the formalin-fixed lungs were stained with hematoxylin and eosin for estimating inflammation in subepithelial regions or periodic acid-Schiff stain for assessing the abundance of mucin producing cells. Stained sections were analyzed by using a Photometrics CoolSNAP Fx CCD (Tucson, AZ, USA) digital camera mounted on a Nikon Eclipse TE 200 (Tokyo, Japan) fluorescence microscope.

Measurement of mucin levels in BALF samples

MUC5AC levels in BALF were assessed by ELISA. Serial dilutions of BALF were incubated at 37°C in triplicate 96-well plates until dry. After blocking, plates were incubated with biotin-conjugated mouse monoclonal MUC5AC antibody for 1h (1:10,000 dilutions) (Lab Vision, Fremont, CA, USA). Following the washing steps the plates were incubated with streptavidin-horseradish peroxidase goat anti-mouse IgG conjugate (1:10,000) for 1 h. To obtain the colorimetric product plates were incubated with peroxidase substrate (3,3',5,5'-tetramethylbenzidine), which was quantified at 450 nm.

Assay for total antioxidant potential of murine airways

To measure total antioxidant capacity of the airways, naïve mice were intranasally challenged with RWPE or PBS immediately preceding the exposure to SMF or sham field for 30 min. Bronchoalveolar lavage was performed 15 min after treatment. The BALF samples were centrifuged and then the total antioxidant potential of BALF samples was measured in the supernatants spectrophotometrically at 570 nm by using a Total Antioxidant Capacity Assay Kit (Abcam, Cambridge, UK).

Measurement of RWPE-generated ROS under cell-free conditions and in cultured epithelial cells

Ragweed pollen proteins (100 µg/ml) and 50 µM H_2DCF-DA were incubated in PBS containing 100 µM NADPH in 2 ml final volume in 6-well plates. Regular PBS solution containing 50 µM H_2DCF-DA and 100 µM NADPH was applied as control. A549 cells grown in 6-well plates were loaded with 50 µM H_2DCF-DA at 37°C for 15 min. After removal of the excess probe, cells were treated with PBS containing NADPH or RWPE+NADPH at the concentrations described above. Then the plates were placed into the sham fields or SMF device for 30 min either at lower or upper position. Changes in DCF fluorescence intensity were assessed in a Synergy HT micro plate reader at 488 nm excitation and 530 nm emission.

Skin prick tests on human volunteers

The study population consisted of 62 volunteers (21 males and 41 females, age between 22 and 50 years). All participants provided written informed consent. The placebo-controlled, double-blind, randomized human study was approved by the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center and conducted

at the Department of Pulmonology of the University of Debrecen under the supervision of a specialist.

The tests were performed simultaneously on both inner forearms of the volunteers. All participants were tested with positive control (histamine), negative control (saline) and with 4 aeroallergens (house dust mite, cat fur, mixed grass pollen, and ragweed pollen; all from ALK-Abello, Hørsholm, Denmark). Immediately after introduction of the identical test materials into the skin of both inner forearms, one forearm of the volunteers was exposed to SMF while the other one was exposed to sham field. The wheal reaction was measured immediately after a 15 min exposure period.

Statistical analysis

In the study on pollen-exposed DCs' functions, 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.

In the experiments related to the effects of SMF on allergic inflammation, one-way ANOVA was used to reveal significant differences between multiple groups for normal sample population. For post hoc analysis, Games-Howell tests were applied between pairs of data series, partly because this test is insensitive to unbalanced data size. In case of non-normal populations (like the endpoint titer values), Kruskal-Wallis tests were carried out for the post hoc binary comparisons. Differences were considered to be statistically significant at $p < 0.05$.

4. Results

4.1 Ragweed pollen grains induce oxidative stress in human monocyte-derived DCs

Previous studies have indicated that RWPs possess NAD(P)H oxidase activity, which generates reactive oxygen radicals, thus we tested whether exposure to pollen grains would increase the intracellular level of ROS in cultured monocyte-derived DCs. Pollen exposure rapidly increased the level of intracellular redox sensitive dye (DCF) fluorescence, which could be prevented by heat treatment of the pollen grains. Pretreatment of RWPs with DPI, also significantly decreased the elevation of intracellular ROS level. The presence of antioxidant agent, PBN in the cell culture medium did not significantly change the basal level of intracellular DCF fluorescence; however it attenuated the ragweed pollen-induced oxidative stress in DCs. 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.

4.2 Pollen-induced oxidative stress influences the cytokine production and cell surface marker expression by DCs

It was shown previously that ROS either directly or via oxidatively modified glycoproteins are able to provoke 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 chemokine (IL-8) and proinflammatory cytokine (TNF-α, IL-6) secretion.

After 24 h of the pollen exposure, we found that the levels of released IL-8, TNF-α and IL-6 were significantly higher in the supernatant of pollen-treated DCs than in those of unstimulated cells. The antioxidant (PBN) decreased the amounts of IL-8, TNF-α and IL-6 released by pollen-exposed DCs to the basal levels. We also found that RWP^{H} was not able to completely inhibit the mediator release. 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. In our experiments we used the equivalent amount (16 pg/ml) of LPS from *Escherichia coli* as a control. Treatment of immature DCs with this amount of LPS could not induce IL-6, IL-8 or TNF-α release from the cells.

Treatment of iDCs with pollen grains resulted in a slight increase in the expression of CD40; while the expression of CD80, CD86, CD83 and HLA-DR was markedly upregulated. 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. The

presence of PBN prevented the phenotypic shift of maturation triggered by pollen administration. The low concentration of LPS was not efficient to upregulate the costimulatory and maturation markers on the surface of DCs, thus they remained at an immature state.

4.3 Pollen-derived ROS influence the functions of DCs

4.3.1 Pollen-induced oxidative stress alters the allostimulatory capacity of DCs

DCs are considered as the most potent antigen presenting cells; therefore, in our further experiments we studied the allostimulatory capacity of pollen-primed DCs. Pollen-treated DCs had a strong stimulatory effect on T cell proliferation compared to iDCs. We found that treatments with both heat-treated RWPs and RWP+PBN decreased the allostimulatory capacity of DCs. Stimulation of DCs with LPS (16 pg/ml) resulted in a moderated proliferative response of allogeneic T cells. As positive control, phytohaemagglutinin (PHA) stimulated naïve $CD4^+$ T lymphocytes were used. These results provide evidence that the allostimulatory capacity of pollen-treated DCs depends, at least partly, on oxidative stress induced by pollen exposure.

4.3.2 Pollen-derived ROS change the T cell polarizing cytokine production by DCs

Because IL-12 and IL-10 play a pivotal role in the T helper cell polarizing activity of DCs, next we examined the production of these cytokines by DCs at 24 h after pollen administration. Immature DCs secreted very low amounts of IL-12(p70) and administration of pollen grains in the presence or absence of PBN or treatment with heat-inactivated pollen grains had no significant effect on basal IL-12 release. Pollen-treated DCs produced higher level of IL-10 than untreated DCs. 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. In control experiments, IL-10 production of DCs was not affected by treatment with LPS (16 pg/ml).

4.3.3 Pollen-induced oxidative stress modify the T cell polarizing capacity of DCs

To examine the role of pollen NAD(P)H oxidases in the T cell polarizing capacity of dendritic cells, the cytokine secretion profile of T lymphocytes isolated from peripheral blood of ragweed allergic and non-allergic subjects was analyzed after priming with pollen-exposed autologous DCs. T lymphocytes from ragweed allergic subjects released significantly more IL-3 after priming with immature autologous DCs than those isolated from non-allergic subjects. 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. The level of GM-CSF was also higher in the supernatant of T cells primed with pollen-treated autologous DCs as compared to cells cocultured with untreated autologous DCs. 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; its production was significantly higher in the supernatant of T lymphocytes isolated from ragweed allergic individuals and cocultured with heat-inactivated pollen-exposed autologous DCs. T cells from non-allergic subjects produced higher amount of IFN-γ after priming with pollen-treated autologous DCs, compared to those from allergic donors.

4.4 Characterization of IL-10 producing T cells after coculturing with pollen-primed DCs

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. Priming T cells with iDCs increased the proportion of $CD25^{+}Foxp3^{+}$ T cells. After stimulation with pollen-exposed DCs the ratio of this T cell population was higher, while priming with heat-inactivated-pollen-treated DCs further increased the rate of $CD25^{+}Foxp3^{+}$ T cells. Simultaneous staining for CD25, intracellular IL-10 and Foxp3 identified a low ratio of IL-10-producing cells. Priming with pollen-treated DCs did not change the ratio of $IL\text{-}10^{+}$ T cells as compared to the untreated ones. However, stimulation with heat-inactivated-pollen-exposed DCs increased the proportion of $IL\text{-}10^{+}$ T cells. 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.

4.5 Static magnetic field exposure did not affect the sensitization phase of pollen allergy in mice

Our observations described above suggest that RWP-derived oxidative stress is able to influence DCs' functions during the sensitization phase of allergic reactions. Because SMF is considered as a factor which is capable to modify the ROS levels in biological systems, next we examined the effects of SMF exposure on pollen induced allergic airway inflammation in sensitized mice. In our pilot test we investigate whether SMF-exposure has an effect on the sensitization or elicitation phase of RWPE-induced allergic reactions. Daily exposure to SMF during the sensitization phase prior the intranasal RWPE challenge did not affect the

accumulation of eosinophils in the BALF compared to sham-exposure. On the contrary, a remarkable but statistically not significant decrease in eosinophil count was detected when mice were exposed to SMF on 3 consecutive days after the intranasal challenge. These observations indicated that SMF-exposure did not affect the sensitization phase of the allergic responses and prompted us to examine the effect of SMF-exposure in the elicitation phase in more detail and to increase the number of animals per group for a higher statistical power.

4.6 Exposure to SMF during the elicitation phase decreased allergic airway inflammation in RWPE-sensitized mice

In the full experimental series mice were sensitized with RWPE, challenged with RWPE or PBS, and exposed to SMF or sham field. Exposure to SMF for a single 30 min time period immediately after the intranasal challenge induced a moderate, but significant decrease in total cell counts in BALF, strongly and significantly decreased number of eosinophils in BALF, and lowered the infiltration of inflammatory cells into the subepithelial area of the airways compared to RWPE-challenged, sham-exposed animals. A single SMF-exposure also decreased the RWPE challenge induced MUC5AC levels in BALF to some extent and epithelial cell metaplasia in the airways. Significantly lower numbers of total cells and eosinophils in the BALF together with decreased accumulation of inflammatory cells in the subepithelial area were also detected upon exposure to SMF for 30 min on 3 consecutive days following RWPE challenge. This 3x30 min SMF-exposure significantly reduced MUC5AC levels in BALF and markedly lessened epithelial cell metaplasia in the airways as compared to sham-exposed animals. Prolonged exposure to SMF (60 min a day) on 3 consecutive days after RWPE challenge further decreased the total cell and eosinophil numbers in the BALF, and lowered inflammatory cell accumulation in the subepithelial regions of the airways as well. Furthermore, the 3x60 min exposure to SMF was more effective in decreasing MUC5AC levels in the BALF and to lower epithelial cell metaplasia in the airways than the 3x30 min treatment.

4.7 Investigation of the SMF-exposure on ROS production by RWPE under cell-free conditions

To investigate the mechanism behind the observed inhibitory effect of SMF-exposure on allergic airway inflammation, we tested whether SMF-exposure was able to decrease the ROS production by RWPE under cell-free conditions. Sham-exposed RWPE converted the redox-sensitive H_2DCF-DA into DCF leading to 75 times higher fluorescence intensity than that of

PBS control. However, exposure to SMF for 30 min either at lower or upper position did not alter DCF fluorescence signals induced by RWPE.

4.8 SMF-exposure diminished RWPE-induced increase in the ROS levels in cultured epithelial cells

Next, we studied the effect of SMF-exposure on intracellular ROS levels in cultured airway epithelial cells. Addition of RWPE to A549 cells increased the intracellular DCF fluorescence signals compared to PBS treatment. The increase in intracellular ROS levels could significantly be diminished, when the cells were exposed to SMF at the lower position and also, to a smaller extent, when they were in the upper position. Exposure to SMF, either at lower or upper position, did not cause significant changes in levels of intrinsic ROS in PBS-treated cells.

4.9 Exposure of mice to SMF immediately after intranasal RWPE challenge lowered the increase in the total antioxidant capacity of the airways

Based on the results of our cell culture studies we sought to test, whether SMF-exposure could decrease ROS levels in the airways of RWPE-treated mice. To do so, total antioxidant capacity of the BALF samples collected from naïve mice challenged intranasally with RWPE or PBS and exposed to SMF or sham field was determined. Intranasal challenge with RWPE induced a statistically significant increase in the antioxidant capacity of the BALF samples when compared to PBS challenge. Immediate exposure to SMF for 30 min after intranasal challenge significantly lowered the increase in the total antioxidant capacity of the airways induced by RWPE treatment. These findings suggest that effects of SMF-exposure on allergic inflammation are mediated at least partially by the modulation of ROS levels in the airways.

4.10 SMF-exposure had no effect on provoked mast cell degranulation in human skin

Several lines of evidence indicate that ROS play an important role in the regulation of various mast cell responses. To reveal the direct effects of SMF-exposure on mast cell degranulation, human skin prick tests were performed. A statistically significant effect of the SMF-exposure decreasing edema diameter could only be detected in case of the positive control, histamine. Although all allergens, but house dust mite provoked edema showed a tendency to decrease the diameter upon SMF-exposure. These results indicate that SMF-exposure can result in significant reduction of histamine-induced edema formation, while it performs only a weak direct impact on provoked mast cell degranulation.

5. Discussion

Ragweed pollen is one of the most important sources of the aeroallergens in many countries since it is responsible for the majority and most severe cases of seasonal rhinitis, conjunctivitis, and allergic asthma. 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. In our work we examined the role of ROS generated by pollen NAD(P)H oxidases both in the sensitization and elicitation phase of allergic inflammation.

We found that 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. Furthermore, our data are also consistent with the more recently observed oxidative stress induction effect of SPPs on monocyte-derived DCs. Our observation that heat pretreatment, which eliminates pollen NAD(P)H oxidase activity, 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.

Oxidative stress activates the NFκB and MAPK signaling pathways that are responsible for transcriptional activation of proinflammatory cytokine and chemokine genes in DCs. 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 oxidative 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 which has indicated that superoxide anions generated by the reaction of xanthine oxidase on xanthine induce phenotypic maturation of DCs by upregulating CD80, CD83 and CD86 markers. In addition to the increased expression of costimulatory molecules, superoxide anion-treated DCs exhibit enhanced capacity to trigger T cell proliferation.

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. 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 semi-mature DCs.

Previous data showed that oxidative stress can be induced in cultured epithelial cells through the direct contact with pollen grains. 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. This can be explained the fact that DCs in the cell cultures were exposed to different levels of ROS depending on their distance from the pollen grains. Thus during the analysis of the T cell polarizing capacity of pollen-treated DCs, naïve 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 naïve T lymphocytes into effector cells with a mixed profile of cytokine production. 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. We propose that DCs exposed to heat-treated pollen grains are more tolerogenic than pollen stimulated ones, which further confirms the important role of pollen NAD(P)H oxidases in DC activation.

In our work we demonstrated 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 antigens.

While many aspects of pathogenesis of allergic inflammation are well-defined, most of the treatments are symptomatic. Previously, it has been shown that intrapulmonary administration of ROS scavengers can block the ragweed pollen-induced allergic airway inflammation in sensitized mice. However, this phenomenon only occurs when the antioxidants are co-administered with RWPE or applied within a tight time frame after RWPE challenge. These findings suggest that a sustained increase in antioxidant potential in the airways may be a novel therapeutic strategy to attenuate pollen-induced allergic airway inflammation. Because SMF can act on free radicals we have investigated whether SMF exposure has an effect on the sensitization or elicitation phase of pollen-induced allergic reactions in sensitized mice.

We found that daily SMF-exposure during the sensitization phase of RWPE-induced allergic reactions did not modify the intensity of the developing airway inflammation.

Next we focused on the elicitation phase of the allergic reaction. It is known that challenge with RWPE induces an initial neutrophil recruitment that is followed by eosinophil influx in the airways and both cell types contribute to oxidative stress during allergic inflammation. Furthermore, as a response to oxidative stress elevated antioxidant capacity of the airways becomes also detectable. We have found that a single SMF-exposure immediately

after intranasal RWPE challenge down modulated the increase in antioxidant capacity and also lowered allergic inflammation. These findings suggest that SMF-exposure is able to attenuate initial oxidative stress elicited by pollen NAD(P)H oxidases. In addition, repetitive exposure to SMF on 3 consecutive days, starting at 6 h after intranasal RWPE challenge by the time the initial oxidative burst had been abolished also inhibited allergic airway inflammation.

Next we investigated the mechanism of action of SMF on ROS. We found that SMF-exposure was not able to modify ROS production by NAD(P)H oxidases in RWPE in cell-free solution. Concerning magnetic spin effects, SMF-exposure can alter those biochemical reactions that involve more than one unpaired electron. It has been shown that the enzyme activity of B_{12}-dependent ethanolamine ammonia lyase changes with SMF-exposure of 100 mT. Experiments have also been carried out with the heme enzymes, horseradish peroxidase, and cytochrome P-450. The exact components, structure, and the mechanism of the enzymatic reaction of pollen NAD(P)H oxidases have not been fully determined yet; therefore, no previous studies have investigated the parameters of superoxide generation by these enzymes under SMF-exposure.

In our cell culture experiments SMF-exposure dampened the increase in intracellular ROS levels in RWPE-treated, cultured A549 epithelial cells. Although the molecular mechanisms of the antioxidant effects of SMF-exposure in our cell culture experiments remain to be explicated, our observations are consistent with a recent study demonstrating that SMF-exposure decreases externally induced oxidative stress modulating activities of antioxidant enzymes including superoxide dismutase, glutathione peroxidase, and catalase in mice fibroblasts.

In order to interact with mast cells the allergen must penetrate into the epithelium during exposure to natural pollen- or subpollen particles. To reveal the direct effects of SMF-exposure on mast cell degranulation, skin prick tests were performed in which small scratches allow the allergens to enter the skin and activate mast cells instead of relying on enzyme activities present in allergy provoking material. In these tests histamine was utilized as positive control, because its injection into the skin by prick technique mimics the allergen-induced edema formation. The results of our human study showed that SMF-exposure significantly decreased edema diameter in response to histamine in agreement with data obtained in an animal model, in which application of SMF-exposure of moderate field strength (5-100 mT) for 15 or 30 min immediately following histamine injection into hind paws resulted in significant edema reduction. Our findings that SMF-exposure was not able to significantly decrease edema formation, triggered by extracts of ragweed and grass pollens

both possessing NAD(P)H oxidase activity, can be explained by previous observations that ROS generated by pollen grains-associated NAD(P)H oxidase have no direct impact on IgE-mediated mast cell degranulation.

Although the relevance of our study to human allergic airway inflammation remains incidental, our data suggest that effects of the SMF gradients around MRI systems, mainly around open field MRI to which our experimental arrangement resembles, should be more carefully investigated, because exposure to SMF including stray field components of MRI may have beneficial effects on pollen-induced allergic conditions.

6. Summary

Ragweed pollen (RWP) is one of the most important sources of aeroallergens in Central European countries because it is responsible for the majority and most severe cases of seasonal rhinitis, conjunctivitis, and allergic asthma. While many aspects of the pathogenesis of these allergic disorders are well defined, the initial steps are not fully understood.

In the present work, first we investigated the effects of reactive oxygen species (ROS) produced by RWP on monocyte-derived dendritic cells (DCs), because DCs play pivotal role in the sensitization phase of allergic responses. Our findings show that exposure to RWP induces an increase in the intracellular ROS levels 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 ROS triggered the production of IL-8, as well as TNF-α and IL-6. Treatment with pollen grains initiated the maturation of DCs, strongly up-regulated the membrane expression of CD80, CD86, CD83 and HLA-DR. The pollen-treated DCs induced the development of naïve 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.

Several lines of evidence demonstrate that oxidative stress contributes to the development of allergic inflammation. Because it has previously been reported that static magnetic field (SMF) acts on biological systems partly through mediating ROS levels, next we investigated the impact of moderate SMF in a murine model of allergic inflammation and also in human provoked skin allergy. We found that even a single 30-min exposure of mice to SMF immediately following intranasal ragweed pollen extract (RWPE) challenge significantly lowered the increase in the total antioxidant capacity of the airways and decreased allergic inflammation. Repeated (on 3 consecutive days) or prolonged (60 min) exposure to SMF after RWPE challenge decreased the severity of allergic responses more efficiently than a single 30-min treatment. SMF-exposure did not alter ROS production by RWPE under cell-free conditions, while diminished RWPE-induced increase in the ROS levels in A549 epithelial cells. Results of the human skin prick tests indicated that SMF-exposure had no significant direct effect on provoked mast cell degranulation.

Our findings draw the attention to the importance of pollen-derived oxidative stress in both sensitization and elicitation phases of allergic inflammation, and may contribute to the development of novel therapeutic approaches.

7. Publications

Register number: DEENKÉTK/97/2014.
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Candidate: Anikó Csillag

Neptun ID: XTMLWO

Doctoral School: Doctoral School of Molecular Cell and Immune Biology

Mtmt ID: 10034595

List of publications related to the dissertation

1. **Csillag, A.**, Kumar, B.V., Szabó, K., Szilasi, M., Papp, Z., Szilasi, M.E., Pázmándi, K., Boldogh, I., Rajnavölgyi, É., Bácsi, A., László, J.F.: Exposure to inhomogeneous static magnetic field beneficially affects allergic inflammation in a murine model.
   J. R. Soc. Interface. 11 (95), 20140097-20140097, 2014.
   DOI: http://dx.doi.org/10.1098/rsif.2014.0097
   IF:4.907 (2012)

2. **Csillag, A.**, Boldogh, I., Pázmándi, K., Magyarics, Z., Gogolák, P., Sur, S., Rajnavölgyi, É., Bácsi, A.: Pollen-Induced Oxidative Stress Influences Both Innate and Adaptive Immune Responses via Altering Dendritic Cell Functions.
   J. Immunol. 184 (5), 2377-2385, 2010.
   DOI: http://dx.doi.org/10.4049/jimmunol.0803938
   IF:5.745

List of other publications

3. Pázmándi, K., Magyarics, Z., Boldogh, I., **Csillag, A.**, Rajnavölgyi, É., Bácsi, A.: Modulatory effects of low-dose hydrogen peroxide on the function of human plasmacytoid dendritic cells.
Free Radic. Biol. Med. 52 (3), 635-645, 2012.
DOI: http://dx.doi.org/10.1016/j.freeradbiomed.2011.11.022
IF:5.271

4. Magyarics, Z., **Csillag, A.**, Pázmándi, K., Rajnavölgyi, É., Bácsi, A.: Identification of plasmacytoid pre-dendritic cells by one-color flow cytometry for phenotype screening.
Cytometry A. 73 (3), 254-258, 2008.
DOI: http://dx.doi.org/10.1002/cyto.a.20529
IF:3.259

Total IF of journals (all publications): 19.182

Total IF of journals (publications related to the dissertation): 10.652

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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Oral presentations

Csillag A., Kumar B. V., Szabó K., Pázmándi K., Rajnavölgyi É., László J., Bácsi A.: Exposure to inhomogeneous static magnetic field decreases pollen-induced allergic airway inflammation in a murine model *6th Molecular Cell and Immune Biology Winter School, 8-11 January 2013, Galyatető, Hungary*

Csillag A.: Egerek és emberek: A Th17 sejtek differenciációja és szerepe az allergiás megbetegedésekben *A Magyar Allergológiai és Klinikai Immunológiai Társaság és a Magyar Immunológiai Társaság XVIII. Továbbképzése 2009. április 24-25. Mezőkövesd*

Csillag A.,. Boldogh I, Pazmandi K., Magyarics Z., Rajnavolgyi E., Bacsi A.: Pollen-grain-induced Oxidative Stress Activates Dendritic Cells *American Academy of Allergy, Asthma & Immunology 2009 Annual Meeting 15 March 2009, Washington DC, USA*

Csillag A., Rajnavölgyi É., Bácsi A.: A pollenszemek által termelt reaktív oxigéngyökök hatása a dendritikus sejtek működésére *A Magyar Immunológai Társaság Ifjúsági Napja, a Magyar Immunológiai Társaság XXXVI. Kongresszusa, 2006. nov. 17., Pécs*

Poster presentations

Csillag A., Kumar B. V., Szabó K., Pázmándi K., Rajnavölgyi É., László J., Bácsi A.: Effect of static magnetic field on pollen-induced allergic airway inflammation in a murine model *Magyar Immunológiai Társaság 42. Vándorgyűlése, 2013. október 16-18., Pécs*

Csillag A., Kumar B. V., Szabo K., Pazmandi K., Rajnavolgyi E., Laszlo J., Bacsi A.: Effect of static magnetic field on pollen-induced allergic airway inflammation in a murine model *15th International Congress of Immunology 22-27 August 2013, Milan, Italy*

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Pázmándi K., Magyarics Z., Csillag A., Rajnavölgyi É., Bácsi A.: A reaktív oxigén gyökök lehetséges szerepe az immunológiai tolerancia kialakításában *Magyar Szabadgyök Kutató Társaság V. Kongresszusa 2009. augusztus 27-29. Szeged*

Csillag A., Boldogh I., Pazmandi K., Magyarics Z., Rajnavolgyi E., Bacsi A.: Pollen-induced oxidative stress alters dendritic cell functions *Allergy & Asthma Symposium: Bridging Innate and Adaptive Immunity 28-29 May 2009, Brugges, Belgium*

Magyarics Z., Csillag A., Pázmándi K., Rajnavölgyi É., Bácsi A.: Identification of plasmacytoid pre-dendritic cells by one-color flow cytometry for phenotype screening *XXIV International Congress of ISAC (Int. Society for Analytical Cytology) 17-21th May 2008, Budapest, Hungary*

Csillag A., Magyarics Z., Rajnavölgyi É., Bácsi A.: A pollen NAD(P)H oxidázok által termelt reaktív oxigéngyökök a dendritikus sejtek működésének megváltoztatásával befolyásolják a veleszületett és a szerzett immunválaszt is *XXXVI. MIT Vándorgyűlés, 2007. október 17-19. Hajdúszoboszló*

Csillag A., Rajnavolgyi E., Bacsi A.: Effect of reactive oxygen species generated by pollen grains on phenotypic and functional features of dendritic cells *2nd ENII-MUGEN Summer School in Advanced Immunology, 14-21 April 2007, Capo Caccia, Italy*

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