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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Abbreviations

APC	- antigen presenting cell
ATP	- adenosine triphosphate
BALF	- bronchoalveolar lavage fluid
BDCA	- blood dendritic cell antigen
BSA	- bovine serum albumin
CAT	- catalase
CCL	- chemokine ligand with C-C motif
CCR	- C-C chemokine receptor
CFSE	- carboxyfluorescein succinimidyl ester
cDC	- conventional dendritic cell
CXCR	- C-X-C chemokine receptor
DAMP	- danger-associated molecular patterns
DC	- dendritic cell
DMEM	- Dulbecco's modified Eagle's medium
DPI	- diphenyleneiodonium
EDTA	- ethylenediaminetetraacetic acid
ELISA	- enzyme linked immunosorbent assay
FBS	- fetal bovine serum
FcεR	- Fc receptors for IgE
GATA3	- GATA binding protein 3
GM-CSF	- granulocyte-macrophage colony-stimulating factor
GSH	- glutathione
GSH-Px	- glutathione peroxidase
GSSG	- oxidized glutathione
H_2DCF-DA	- 2'-7'-dihydro-dichlorofluorescein diacetate
HDM	- house dust mite
4-HNE	- 4-hydroxynonenal
H_2O_2	- hydrogen peroxide
HOCL	- hypochlorous acid
HSP	- heat shock protein
iDC	- immature dendritic cell
IFN	- interferon
IL	- interleukin
ILC	- innate lymphoid cell
LPS	- lipopolysaccharide
LTB_4	- leukotriene B_4
MAPK	- mitogen-activated protein kinase
MCP-1	- monocyte-chemotactic protein-1
MDA	- malondialdehyde
MHC	- major histocompatibility complex
MIP1-α	- macrophage inflammatory protein 1 alpha
MRI	- magnetic resonance imaging
MyD88	- myeloid differentiation primary response gene 88
NADPH	- nicotinamide adenine dinucleotide phosphate
NFκB	- nuclear factor kappa-B
NLR	- NOD-like receptor
NO	- nitric oxide
NOD	- nucleotide binding oligomerization domain

Nrf2	- nuclear regulatory factor 2
$O_2^{\bullet-}$	- superoxide anion
•OH	- hydroxyl radical
$ONOO^-$	- peroxynitrite
PAF	- platelet-activating factor
PAMP	- pathogen-associated molecular patterns
PBMCs	- peripheral blood mononuclear cells
PBN	- N-tert-butyl-a phenylnitrone
PBS	- phosphate buffered saline
pDC	- plasmacytoid dendritic cell
PGD_2	- prostaglandin D_2
PGE_2	- prostaglandine E_2
PHA	- phytohaemagglutinin
PI3K	- phosphoinositide 3-kinase
PRR	- pattern recognition receptor
RANTES	- regulated on activation, normal T expressed and secreted
RFI	- relative fluorescence intensity
RIG	- retinoid acid-induced gene I
RLR/RLH	- RIG-like receptor/helicase
ROS	- reactive oxygen species
RWP	- ragweed pollen grain
RWPE	- ragweed pollen grain extract
RWP^H	- heat-treated ragweed pollen grain
SMF	- static magnetic field
SOD	- superoxide dismutase
SPPs	- subpollen particles
STAT6	- signal transducer and activator of transcription 6
Tfh	- follicular helper T cell
TGF-β	- transforming growth factor-beta
Th	- helper T cell
TLR	- Toll-like receptor
TNF	- tumor necrosis factor
TRx	- thioredoxin
Treg	- regulatory T cell
Tr1	- type 1 regulatory T cell
TSLP	- thymic stromal lymphoprotein
VEGFA	- vascular endothelial factor A

Contents

SUPPLEMENTARY 1

Csillag A, Kumar BV, Szabó K, Szilasi M, Papp Z, Szilasi ME, Pázmándi K, Boldogh I, Rajnavölgyi E, Bácsi A, László JF: Exposure to inhomogeneous static magnetic field beneficially affects allergic inflammation in a murine model.

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SUPPLEMENTARY 2

Csillag A, Boldogh I, Pazmandi K, Magyarics Z, Gogolak P, Sur S, Rajnavolgyi E, Bacsi A: Pollen-induced oxidative stress influences both innate and adaptive immune responses via altering dendritic cell functions.

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1. Introduction

The prevalence of allergic airway disorders, like asthma and allergic rhinitis, has dramatically increased in the last decades in industrialized countries, a phenomenon known as asthma and allergy epidemic. Namely, according to the World Health Organization (WHO), 300 million people suffered from asthma worldwide in 2005 (Bloemen et al. 2007). These diseases are characterized by significant negative effects on personal life quality and induce a considerable socio-economic impact due to health care utilization and treatment costs. As reported by a GA^{2}LEN study, in 2009 approximately 50% of the Hungarian population was sensitized to a highly allergenic plant species, the common ragweed (*Ambrosia artemisiifolia*) (Burbach et al. 2009). 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. Allergic sensitization is a multifactorial process in which exposure to allergens, environmental risk factors, viral respiratory infection history and genetics have a predominant role particularly in the vulnerable period of early life when most of the sensitizations happen. Furthermore, adjuvant activity of airborne allergens also contributes to the enhancement of their antigenicity. After inhalation, allergens can be deposited on the airway mucosal surfaces and are taken up by innate immune cells, such as dendritic cells (DCs) and macrophages. The principal antigen presenting cells, the DCs transport allergens to the T cell area of the draining lymph nodes. Here, the processed antigens are presented to cognate naïve T cells leading to the development of allergen-specific T helper type 2 (Th2) cells. These cells engage cognate B cells inducing them to undergo immunoglobulin class switching and produce IgE antibodies. Then high affinity Fcε receptors (FcεRI), expressed by tissue mast cells and blood basophils, strongly bind IgE; these sets of events result in sensitization of an individual /reviewed in (Galli et al. 2008)/.

The elicitation phase is initiated when allergens re-enter the body and crosslink the bound IgE on the surface of mast cells causing their degranulation, an immediate release of several kind of mediators, such as histamine, tumor necrosis factor-alpha (TNF-α) and a range of enzymes. The release of histamine and other vasoactive compounds increases

vascular permeability and local blood flow, and can act on smooth muscle to increase the expulsion of mucosal allergens. In addition, histamine enhances epithelial cell mucus production, which may aid in allergen immobilization and cytoprotection. These early mediators are responsible for the development of the well-known early allergic symptoms such as sneezing, wheezing, swelling, itching, runny nose and obstructed breathing. Other mast cell products, such as various lipid mediators and cytokines, contribute to the initiation of a facultative late-phase reaction by recruiting and activating eosinophils, neutrophils and Th2 cells, and by interacting with tissue cells such as nerve cells, smooth-muscle cells, endothelial cells and the epithelium /reviewed in (Galli et al. 2008)/.

Many aspects of the pathogenesis of allergic inflammation are well defined, but we have only limited knowledge about the precise mechanisms and factors that influence the initiation of allergic disorders. This work aimed to study the effects of reactive oxygen species (ROS)-producing activity of ragweed pollen grains (RWPs) on human monocyte-derived DCs' functions. Moreover, we have tested a novel approach, exposure to static magnetic field (SMF), to modulate allergic responses in a murine model of allergic inflammation and also in human provoked skin allergy.

2. Theoretical background

2.1 Sensitization phase of airway allergy

In the initiation of allergic airway responses a specialized population of antigen presenting cells (APCs) called dendritic cells play a fundamental role by bridging innate and adaptive immunity. Dendritic cells were first visualized in the basal layer of the skin epidermis by Paul Langerhans in 1868 and were presumed to be the part of the nervous system because of their morphology (formation of long dendrites). DCs remained uncharacterized until 1973, when Steinman and Cohn described a unique population of white blood cells in the mouse spleen with particular features and functions (Banchereau and Steinman 1998). For this discovery, Steinman was awarded Nobel Prize in Medicine and Physiology in 2011.

2.1.1 Localization of DC subsets in the airways

Presence of bone marrow-derived DCs in the trachea, bronchi, alveoli and visceral pleura was firstly described by Sertl and colleagues (Sertl et al. 1986). These DCs were shown to express MHC class II at a high level and have a common dendritic morphology but lack Birbeck granules, typically described in skin Langerhans cells. Furthermore, *in vitro*, they act as potent T cell stimulators (Sertl et al. 1986). In the recent decades following this initial discovery, numerous papers have been published to describe the precise surface phenotype and functions of DCs, including the characteristics of airway DCs.

Based on murine studies DC subsets in the lung can be grossly divided into 2 categories: (1) conventional dendritic cells (cDCs), which express high levels of the integrin CD11c and can be further subdivided into $CD11b^+$ or $CD11b^-$ populations (Wikstrom and Stumbles 2007), and (2) plasmacytoid dendritic cells (pDCs), which express Siglec-H, Ly6C and B220 but low levels of CD11c (Gill 2012; von Garnier et al. 2005). Importantly, these DC subsets have been indentified in specific anatomic locations in the airways with distinct functions (Figure 1).

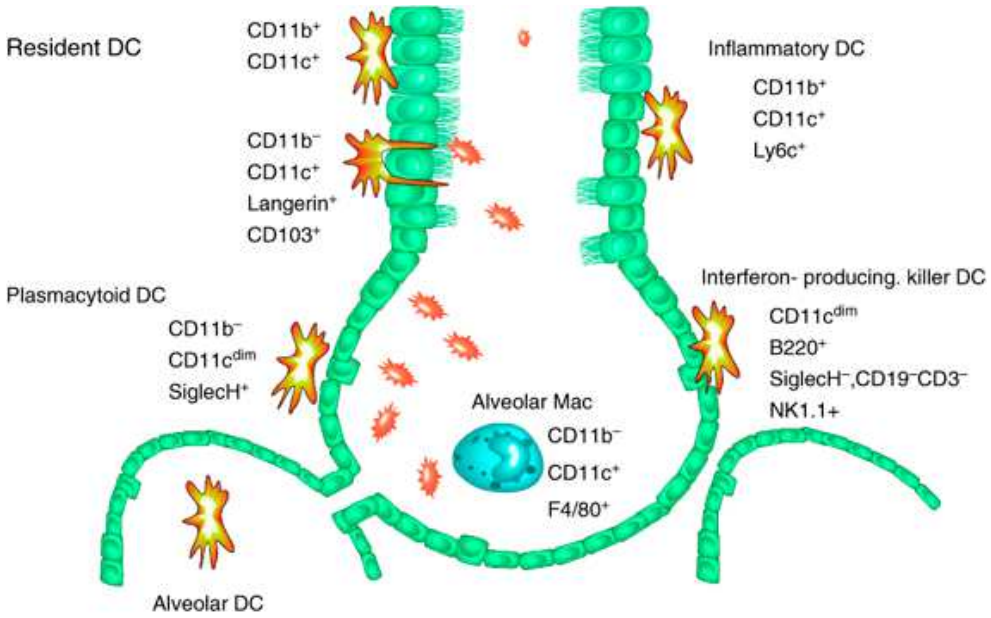

Figure 1. Localization of murine DC subsets in the airways. *Two populations of resident conventional DCs (cDCs) are placed underneath the airway epithelial cell layer. Mucosal CD11b$^-$ cDCs are situated in the basolateral space and can process their dendrites between epithelial cells directly into the airway lumen. CD11b$^+$ resident cDCs and plasmacytoid DCs (pDCs) are located underneath the basal membrane. In the alveolar space, alveolar DCs and macrophages are present. During inflammatory conditions, like respiratory viral infections, an activated population of inflammatory DCs expressing CD11b and Ly6C can be found in the lung tissue as well as the interferon-producing killer DCs (IKDCs) (GeurtsvanKessel and Lambrecht 2008).*

The trachea and large conducting airways contain a well-developed network of intraepithelial DCs which express CD103 but not CD11b. They extend their dendritic projections between epithelial cells allowing to sample airway lumen content, even in steady state conditions (Gill 2012). These CD103$^+$ DCs are also specialized in cross-presentation of exogeneous viral antigen to CD8$^+$ T cells (Desch et al. 2011; GeurtsvanKessel et al. 2008). The other airway DC subset is the CD103$^-$, CD11b$^+$ CD11c$^+$ cDC that 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 (del Rio et al. 2007; van Rijt et al. 2005).

Lung pDCs in steady state conditions 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. After taking up inhaled harmless antigens they drive the formation of regulatory T cells (Tregs) (de Heer et al. 2004).

Upon inflammatory stimuli such as microbial infections or exposure to environmental pollution/allergens, considerable amount of proinflammatory chemokines (for example the CCR2 ligand CCL2 and CCL7) produced by airway epithelial cells attracting more DC progenitors from the bone marrow to the lungs (Hammad et al. 2009; Serbina and Pamer 2006). These $CCR2^{hi}$ monocytes can be the precursors to so-called inflammatory $CD11b^{+}$ $CD11c^{+}$ DCs, which still express the monocytic marker, Ly6C (Robays et al. 2007).

The presence and the distribution of different DC subtypes in the human airways have not been fully elucidated yet, but a distinction can be made between cDCs and pDCs. In humans the $CD11c^{+}$ cDCs can be further divided into 2 groups: type 1 cDCs that express blood dendritic cell antigen (BDCA) 1 also known as CD1c, and type 2 cDCs that express BDCA3 (CD141). The members of the other group, the pDCs express BDCA2 (CD303) and CD123, the receptor for IL-3 (Demedts et al. 2005). Moreover, the presence of another DC type which expresses CD1a was also confirmed in the epithelium of the conducting airways. Interestingly, it was found that although several DCs coexpress CD1a and CD1c, like Langerhans cells in the skin, $CD1a^{+}$ $CD1c^{-}$ as well as $CD1a^{-}$ $CD1c^{+}$ DCs are also present in the human lung (Demedts et al. 2005). A previous study, using DCs differentiated from monocytes *in vitro*, demonstrated that $CD1a^{+}$ and CDa^{-} DC subsets differ in their functional characteristics (Gogolak et al. 2007).

2.1.2 Sensing the antigen/allergen by DCs

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. The issue of antigen capture by intraepithelial DCs seems to be easily solved as these type of DCs are located in the basolateral layer of airway epithelium, separated from the inhaled air only by epithelial cells tightly connecting to each other with several junctional proteins.

Previously, Rescigno and colleagues have shown in mice that DCs reside in the submucosa are able to open the tight junctions between the gut epithelial cells, project their dendrites through the epithelium and directly sample the content of the gut lumen. Considering that DCs express tight junction proteins, the integrity of epithelium is preserved (Rescigno et al. 2001). Similarly, in the airways intraepithelial DCs also bear

tight junction proteins such as claudin 1, claudin 7 and zonula 2 as demonstrated in mice (Hammad and Lambrecht 2008). In the nasal mucosa of individuals suffering from allergic rhinitis HLA-DR^+ $CD11c^+$ DCs express claudin 1 and penetrate through the epithelium, which indicates that DCs easily access antigens (Takano et al. 2005). Nevertheless, a study imaged the functions of airway DCs by using two-photon microscopy queried the primary role of intraepithelial DCs in antigen sampling as antigen accumulation could not be detected in these DCs after *in vivo* exposure of mice with fluorescently labeled antigen. Alternatively, it is suggested that inhaled particles need to breach the epithelial barrier to be detected by DCs (Veres et al. 2011). Indeed, some enzymatic and non-enzymatic properties of allergens are able to break off epithelial integrity and additional environmental stimuli such as respiratory viruses and air pollutants (for example ozone, diesel exhaust particulates and cigarette smoke) may also contribute to this phenomenon (Hackett et al. 2011).

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 the endogenously generated danger signals (DAMPs) during antigen capture by DCs. The PAMPs such as bacterial, fungal and viral products are recognized by membrane-bound, cytoplasmic or vesicular (endosomal) pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and retinoid acid-induced gene I (RIG)-like receptors or helicases (RLRs or RLHs) (Holgate 2012). Signaling through myeloid differentiation primary response gene 88 (MyD88), a common TLR adaptor molecule, strongly activates DCs to increase the expression of costimulatory molecules (CD80 and CD86) and to produce pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-1, IL-6 and IL-12 (Eisenbarth et al. 2002; Piggott et al. 2005). Low dose of TLR ligands from microbes in house dust, when inhaled along with allergens, provides the necessary adjuvant effect to drive allergic responses (Holgate 2012). Furthermore, activation of selective TLRs on epithelial cells enhances DC motility and antigen sampling through the production of Th2-promoting chemokines (CCL17 and CCL22 via CCR4) and cytokines (IL-25, IL-33, granulocyte-macrophage colony-stimulating factor; GM-CSF and thymic stromal lymphopoietin; TSLP) (Lam et al. 2008). Indeed, TLR4 expression on lung structural cells is necessary and sufficient for driving activation of the mucosal DCs and determines the development and severity of allergic inflammation

following house dust mite (HDM) exposure (Hammad et al. 2009). Other PRRs sense DAMPs, including heat shock proteins (HSPs), adenosine triphosphate (ATP), uric acid and ROS, produced during tissue damage (Willart and Lambrecht 2009). These DAMP molecules contribute to the induction of inflammation by recruiting and activating innate inflammatory cells. It was firstly demonstrated by Idzko et al. that an endogenously released danger signal, ATP had a crucial role in promoting Th2 sensitization by lung DCs. Administration of ATP analogs induced a break in inhalation tolerance by upregulating the expression of costimulatory molecules and promoting migration through increased CCR7 expression. They also showed that exposure of asthmatics to HDM allergen induces an acute release of ATP into the bronchoalveolar lavage fluid (Idzko et al. 2007).

It was also reported that ROS enhanced the capacity of DCs to promote $CD4^+$ T cell activity demonstrated by an increase in their proliferation and production of IFN-γ, IL-6 and IL-2 and reduced Treg generation by these DCs. It was also demonstrated that oxidative stress activates phosphoinositide 3-kinase (PI3K) and nuclear factor kappa-B (NFκB) pathways and increases DC migration (Batal et al. 2014).

To sum it up, in the absence of TLR ligation DCs remain relatively inactive, thus PRRs, due to their crucial adjuvant sensing role, are of great importance in directing allergen sensitization.

2.1.3 Role of adjuvant activities of allergens in sensitization

Not only endogenous but exogenous danger signals, which can be associated with the allergen itself, are capable to initiate the activation of DCs and in 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. Airborne allergens derived from different sources such as HDM, cockroach, fungi or pollen were identified to possess cysteine, serine or aspartic proteases (Gunawan et al. 2008; Holgate 2012). Der p 1, a major allergen from the HDM (*Dermatophagoides pteronyssinus*) shows cysteine protease activity that can directly activate DCs or epithelial cells, and contribute to the development of Th2 sensitization processes (Hammad et al. 2001).

Furthermore, pollen grains liberate bioactive lipids, the so-called pollen-associated lipid mediators. The aqueous extracts from birch pollen consist of predominantly E1-

phytoprostanes, resembling the structure and function of prostaglandin E_2 (PGE_2). These mediators are able to inhibit the IL-12 production of DCs by blocking NFκB translocation and change the migratory capacity of immature DCs through upregulation of CXCR4 and downregulation of CCR1 and CCR5 expression. All these alterations in DCs' functions contribute to the development of Th2-dominated immune response; however, this effect cannot solely be attributed to the pollen associated lipid mediators (Gilles et al. 2009; Mariani et al. 2007; Traidl-Hoffmann et al. 2005).

More recently, the pollen metabolom was further analyzed for candidate immunomodulatory substances by that research group. They found that fractions of aqueous pollen extracts comprised adenosine in μM concentrations which was able to induce cAMP via A2 receptor dependent manner and inhibit IL-12 production by DCs. Pollen-associated adenosine contributes to the inhibition of Th1 responses and the induction of Tregs (Gilles et al. 2011).

Additionally, pollen grains contain NAD(P)H oxidase, an enzyme that generates ROS providing a signal for the initiation of allergic inflammation in mice (Boldogh et al. 2005). The function of pollen NAD(P)H oxidases will be detailed in Section 2.3 describing the relationship between oxidative stress and allergic inflammation in the airways.

Finally, it should be noted that air pollution might influence pollen allergenicity thus immune cell activation. It was reported that upon exposure to ozone the amount of NAD(P)H oxidase was elevated in ragweed pollen extracts (Pasqualini et al. 2011). In another paper, it was demonstrated that high levels of ozone are associated with higher allergen expression but less pollen-associated lipid mediators thus these pollens rather have immuno-stimulatory than immuno-modulatory potential (Beck et al. 2013).

2.1.4 T cell polarization in the lymph node

After sensing and taking up antigens at mucosal surfaces, lung DCs migrate via the afferent lymphatics to the draining lymph nodes (Vermaelen et al. 2001) and present the processed antigens to T cells and initiate cognate T cell responses. This migration process happens in a manner dependent on CCR7. Among different DC subsets in the lung, $CD103^+$ and $CD11b^+$ cDCs upregulate CCR7 expression (while CXCR1, CCR1 and CCR5 expression is downregulated) react to chemokines CCL19 and CCL22 and migrate to lymph nodes to initiate Th2 cell responses.

DCs are crucial in regulating immune responses by bridging innate and adaptive immunity since they translate information originated from antigens to naïve T cells providing them their first activation signals and promoting their commitment to Th1, Th2, Th17 or Treg directions. 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). Sensitization steps to allergens are depicted on Figure 2.

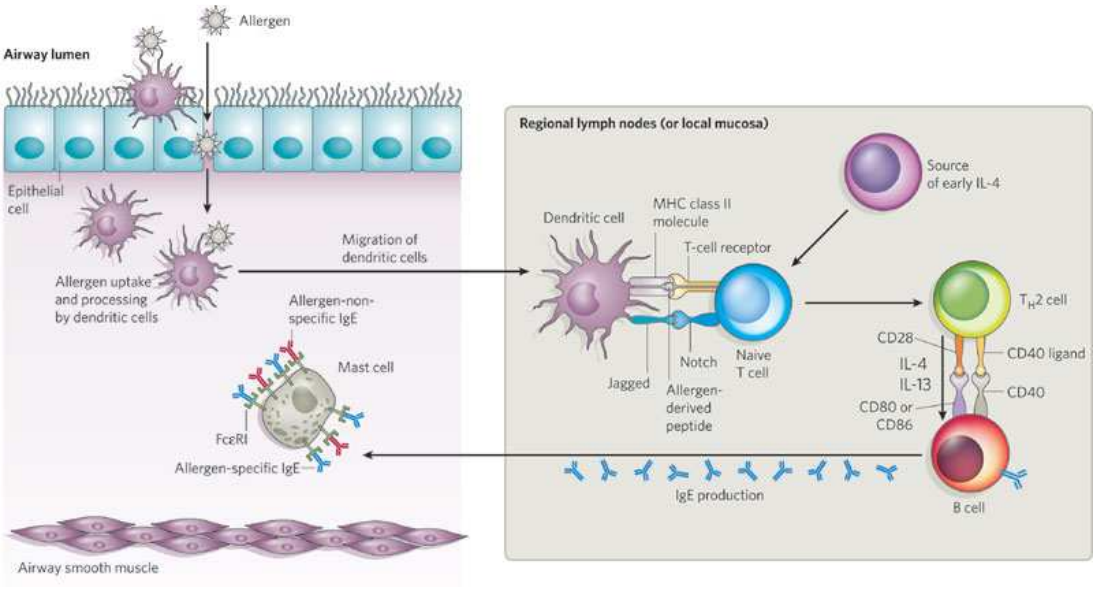

Figure 2. Sensitization to allergens in the airway. *Allergen can be taken up by dendritic cells in the mucosa of the airways. Then, dendritic cells migrate to regional lymph nodes where they present peptides derived from the processed allergen to naïve T cells through MHC class II molecules. In the presence of early IL-4 and costimulators on dendritic cells naïve T cells differentiate to Th2 cells which produce IL-4 and IL-13. In the presence of these cytokines and the ligation of suitable co-stimulatory molecules B cells undergo isotype switching process resulting in IgE production. The IgE binds to the high-affinity FcεRI on tissue-resident mast cells, thereby sensitizing them to respond when the host is later re-exposed to the allergen (Galli et al. 2008).*

One major determining factor in the polarization of naïve $CD4^+$ T cells is the cytokine microenvironment; instructive cytokines such as IL-4, IL-12, IL-10, IL-6 and transforming growth factor-beta (TGF-β) are known to participate in Th cell differentiation, and most of them are produced by DCs.

Neither steps of initial allergen sensitization, nor the mechanisms responsible for the differentiation of naïve $CD4^+$ T cells into Th2 cells are well understood. It is generally

thought that the cytokine milieu directs the differentiation of naïve $CD4^+$ T cells into various Th subsets. Several lines of evidence indicate that Th2 polarization requires an initial source of IL-4 to activate the signal transducer and activator of transcription 6 (STAT6) which induces the expression of the key transcription factor GATA binding protein 3 (GATA3) and drives the production of Th2 cytokines (Paul and Zhu 2010). Two competing ideas have emerged to explain the cellular source of the IL-4. The first one suggests that the Th2 responses develop by default in the absence of strong Th1 and Th17 promoting cytokines or when the strength of MHC class II and T cell receptor interaction is weak in the immunological synapse. In this model the naïve $CD4^+$ T cells produce the instructive IL-4 (Hammad et al. 2010). In another view, IL-4 was presumed to be produced by accessory cells, such as natural killer T cells, eosinophils, mast cells or basophils (Paul and Zhu 2010). It was demonstrated that, in parasite infected or papain injected mice, basophils migrate into the lymph nodes draining the site of the antigen exposure and at the same time act as an effective APC and release IL-4 and TSLP in response to allergens (Sokol et al. 2008; Yoshimoto et al. 2009). In the protease allergen-induced model of Th2 cell differentiation, basophils seem to be necessary and sufficient as APCs for Th2 induction, meanwhile DCs are apparently dispensable for this process (Sokol et al. 2008). However, for inducing Th2 immunity to inhaled allergens the similar importance of basophils has not been fully elucidated. In fact, inflammatory DCs have indispensable role for priming Th2 rather than basophils (Hammad et al. 2010). Notably, the recently discovered group 2 innate lymphoid cells (ILC2s) in the airway mucosa are likely to be the rapid and potent source of the type 2 cytokines IL-5 and IL-13 (Halim et al. 2012) and thus are able to influence the downstream adaptive Th2 cell response. Intranasal administration of the protease-allergen papain stimulated ILC2s and Th2 cells leading to the development of allergic lung inflammation and elevated IgE titers. Furthermore, IL-13 produced by ILC2 cells was critical for promoting the migration of activated lung DCs to the draining lymph nodes where they induce the differentiation of naïve $CD4^+$ T cells into Th2 cells while IL-4 was not required for papain-induced Th2 cell differentiation (Halim et al. 2014).

It has been proposed that other cytokines such as IL-6 could be decisive in Th2 instruction (Dodge et al. 2003; Krishnamoorthy et al. 2008; Rincon et al. 1997), since IL-4 KO mice can still mount a Th2 response (Okahashi et al. 1996).

Costimulatory or accessory molecules on DCs are also required to ensure T cell division and differentiation into effector cells. Activation of GATA3 and STAT6, which

is required for Th2 differentiation, can be upregulated by Jagged-Notch signaling (Amsen et al. 2007). Notch dependent signals have been shown to directly regulate GATA3 expression and critically control Th cell fate *in vivo* (Amsen et al. 2004; Fang et al. 2007). However, it has also been suggested that Notch signals provide survival and proliferative advantage to committed Th cells rather than direct commitment to the Th cell fate (Ong et al. 2008). Nonetheless, recently Jagged1, and not Jagged2, expression on DCs has been shown to be critical for IL-4 induction and promotion of allergic responses (Okamoto et al. 2009). Besides the expression of Jagged and production of cytokines, other surface markers on DCs can also influence Th2 differentiation. The expression of OX40L on DCs has been shown to be required for optimal Th2 priming (Jenkins et al. 2007; Wang and Liu 2009). The expression of OX40L on DCs is dependent on signaling through CD40 (Jenkins et al. 2007). The epithelial cell-derived TSLP is also capable to induce OX40L expression in DCs inducing the differentiation of allergen-specific naïve $CD4^+$ T cells to inflammatory Th2 cells (Ito et al. 2005). Besides Th2 cells, additional T cell subsets have been recognized to play important roles in allergic diseases. These include the well-established Th1 and more recently discovered Th9, Th17, Th22, Treg, type 1 regulatory T (Tr1) and follicular helper T (T_{fh}) subsets. As illustrated in Figure 3, cytokines produced by these cells have different functional roles in allergy.

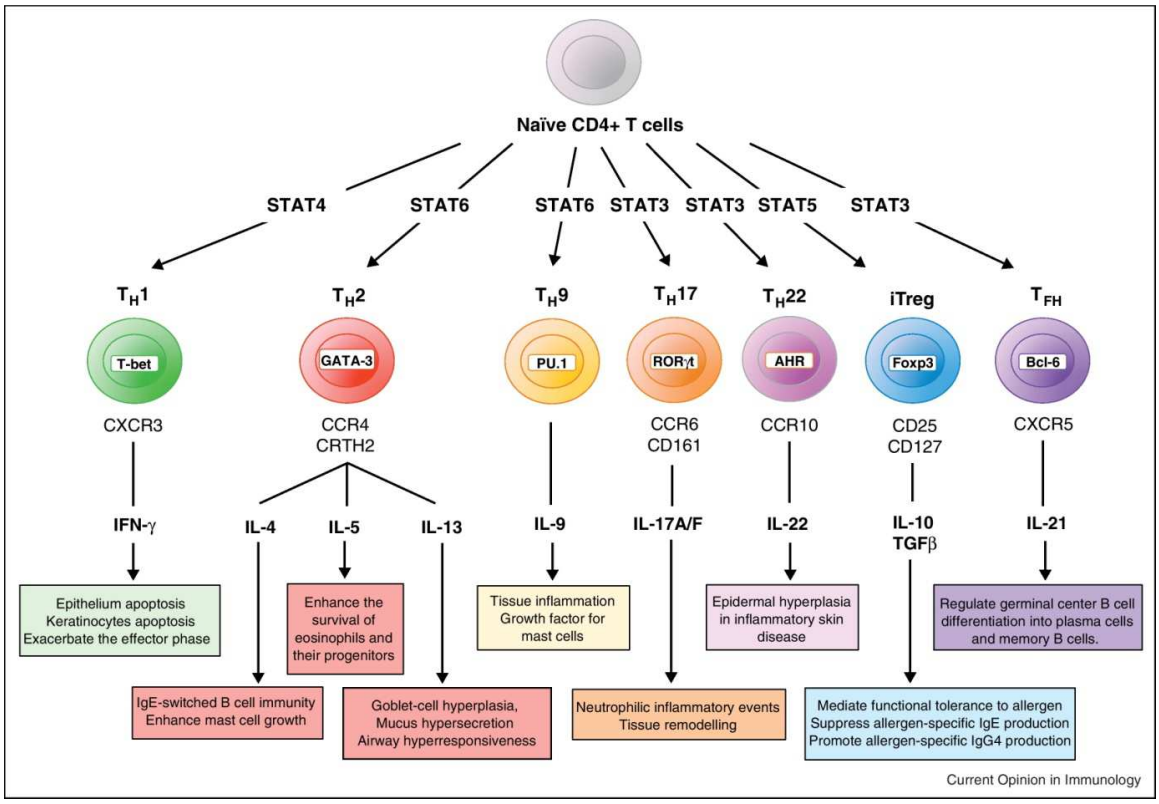

Figure 3. T cell subsets in allergic diseases. *Diverse T cell lineages differentiated from naïve T cells can be defined based on characteristic transcription factors, surface markers and effector cytokines. Each T cell subsets has a demonstrated role in either promoting or suppressing allergic diseases (Wambre et al. 2012).*

Taken together, the above mentioned signals promote the allergen activated Th2 cells to 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 FcεRI on the surface of mast cells and peripheral blood basophils resulting to become „armed" or sensitized.

2.2 Elicitation phase of allergic inflammation

The elicitation phase of allergic inflammation is often classified into three stages. Early phase reactions are induced within seconds to minutes of allergen challenge, while late-phase reactions occur within several hours. By contrast, chronic allergic inflammation is a persistent inflammation that occurs at sites of repeated allergen exposure.

Re-exposure to the allergen leads to crosslinking of IgE-binding FcεRI receptors on the mast cell and results in the secretion of various biologically active products. Preformed mediators stored in cytoplasmic granules, including biogenic amines (histamine and serotonin in rodents to a much greater extent than in humans), serine proteases (such as tryptase, chymase and carboxypeptidase) proteoglycans (such as heparin and chondroitin sulphate) are released within minutes after antigen exposure. Newly synthesized lipid-derived mediators /for example prostaglandin D_2 (PGD_2), leukotriene B_4 (LTB_4), LTC_4 metabolized from arachidonic acid through cyclooxygenase and lypoxygenase pathway/ and certain cytokines and growth factors (such as TNF-α and vascular endothelial factor A; VEGFA) also released by activated mast cells. Some activated mast cells can release platelet-activating factor (PAF), as well /reviewed in (Galli et al. 2008)/. In addition to FcεRI-mediated signals, alternative mechanisms have been shown to induce mediator release in mast cells. Several lines of evidence suggest that oxidative stress act as a stimulus for mast cell activation and degranulation (Frossi et al. 2004). 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 (Chodaczek et al. 2009; Endo et al. 2011). This phenomenon is likely due to the pollen-induced increased ROS production via mitochondrial respiratory complex III. In addition, mitochondrial dysfunction triggered by pollen exposure leads to enhanced IgE-mediated IL-4 production (Chodaczek et al. 2009).

2.3 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 reactive oxygen species which evolving by the unbalanced antioxidant and ROS scavenging mechanisms. ROS are formed in biological systems by oxidases and in the process of respiration. The term ROS stands for oxygen radicals that have either unpaired electrons like superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical (•OH) thus rendering them extremely reactive or paired electrons like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCL) and peroxynitrite ($ONOO^-$).

In the development of oxidative stress both endogeneous and exogeneous sources of ROS play significant role in the airways.

Inflammatory cells such as macrophages, neutrophils and eosinophils are considered the primary source of endogeneous ROS in the airways. These activated inflammatory cells can generate $O_2^{\bullet-}$ through NADPH oxidase activity, cytosolic xanthine oxidase system and the mitochondrial respiratory chain. Superoxide anions are spontaneously or enzymatically dismutased to H_2O_2 which can interact with transition metals and form •OH. Eosinophils, neutrophils and monocytes contain peroxidases that catalyze the interaction between H_2O_2 and halides leading to the generation of hypohalides, such as HOCl. In addition, ROS may also react with nitric oxide (NO) to form $ONOO^-$ /reviewed in (Holguin 2013)/. These compounds have been shown to amplify the inflammatory airway processes in asthma. ROS production by neutrophils correlates with the severity of airway inflammation (Sanders et al. 1995). Airway macrophages in patients with asthma produce more superoxide anions than those of control subjects. Even the monocytes and eosinophils collected from the blood of asthmatic patients produce more ROS compared with those of control subjects (Calhoun et al. 1992).

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. These environmental pollutants are able to activate cellular oxidases such as NADPH oxidase, xanthine oxidase, p450 cytochrome oxidase and increase intracellular levels of ROS (Becker et al. 2002). Overproduction of ROS can lead to structural cellular damage through the oxidation of proteins, lipids, DNA and carbohydrates and enhance inflammatory reactions.

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 lungs, the main enzymatic reducers are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and thioredoxin (TRx). Among the non-enzymatic reducers we can find mucin, urate, glutathione (GSH), ascorbate, ceruloplasmin, transferrin, vitamin E, ferritin, and small molecules such as bilirubin (Araneda and Tuesta 2012). If cells exposed to low level of oxidative stress they upregulate the production of antioxidants (including SOD, CAT, GSH-Px) by nuclear regulatory factor 2 (Nrf2)-dependent transcriptional activation of antioxidant response

elements (Li et al. 2004). If antioxidants fail to counterbalance the high levels of ROS production or pro-oxidative stimuli, the increasing oxidative stress can trigger intracellular signaling cascades, such as MAPK and NFκB pathways which induce the expression of various pro-inflammatory cytokines (IL-4, IL-5, IL-8, IL-10, IL-13, TNF-α) and chemokines (MIP-1α, MCP-3, RANTES) playing key roles in airway inflammation (Xiao et al. 2003). Eventually, oxidative stress induces cellular toxicity that originates in the mitochondria leading to cell death by apoptosis or necrosis (Riedl and Nel 2008).

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. Pollens are complex biological packages composed of many proteins, some of which possessing enzymatic activities. Weed pollens contain various antigenic proteins which can be classified into four major families: the ragweed Amb a 1 family of pectate lyases; the defensin-like Art v 1 family from mugwort; the Ole e 1-like allergens Pla l 1 from plantain and Che a 1 from goosefoot; and the nonspecific lipid transfer proteins Par j 1 and Par j 2 from pellitory (Gadermaier et al. 2004). In addition to these antigens, it turned out that pollen grain extracts of different plant species, among them numerous highly allergenic weed, tree and grass pollens, have redox activity which indicated intrinsic NAD(P)H oxidase activity (Boldogh et al. 2005). Interestingly, among the investigated pollen extracts the only exception was an extract from pine pollen which lacks ROS-producing activity consistently with previous studies that pine pollens fail to induce allergic sensitization in humans, however they are present in large quantities in the environment (Boldogh et al. 2005; Bousquet et al. 1984; Harris and German 1985). It is well known that pollen antigens can induce allergic inflammation throughout the respiratory tract but most of the pollens are too large (vary from about 10 to nearly 100 μm; ragweed pollen is approximately 20 μm in diameter) to reach the lower airways. Additionally, increased frequency of exacerbation of allergic reactions in sensitive individuals was observed after heavy rainfall, however the levels of airborne pollen grains plunged following thunderstorms (Wark et al. 2002). The explanation of these contradictory facts can be that hydration and hypotonic shock by rainwater leads to the release of subpollen particles (SPPs) from whole pollen grains of respirable size (varying from 0.5 to 4.5 μm) enabling their penetration to the lower airways. It was also demonstrated that SPPs released from ragweed pollen grains retain their predominant allergen, the Amb a 1, as well as NAD(P)H oxidase activity (Bacsi et al. 2006) (Figure 4).

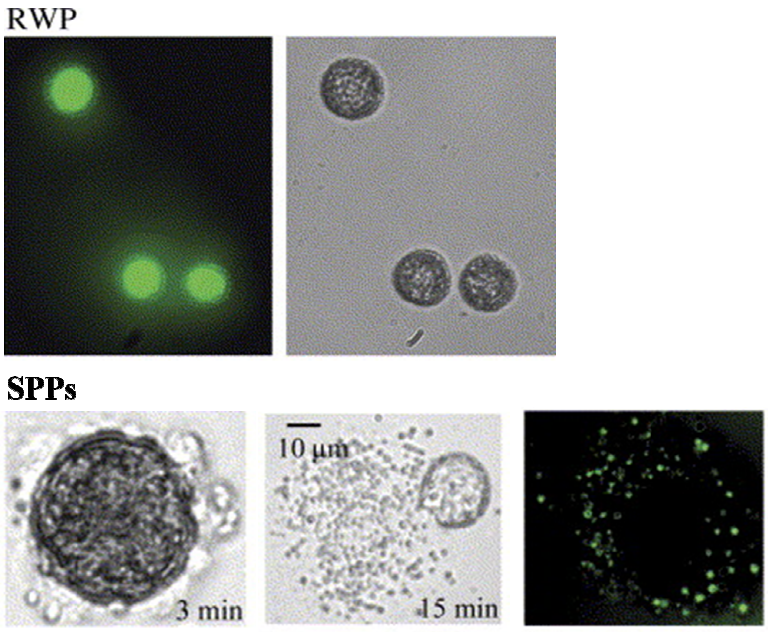

Figure 4. ROS production by ragweed pollen grains (RWP) and subpollen particules (SPPs) released from pollen grains. *To detect ROS formation a redox sensitive green fluorescent dye (DCF) was used.* (Bacsi et al. 2006; Bacsi et al. 2005)

It seems that this pro-oxidant activity of intact hydrated pollen grains, their extracts and the released SPPs play 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. Products generated by oxidative stress such as oxidized glutathione (GSSG) and 4-hydroxynonenal (4-HNE) facilitate allergic airway inflammation induced by pollen antigen (signal 2). Challenging mice with pollen extract that lack NADPH oxidases cannot induce robust allergic inflammation in the lung indicating the necessarity of oxidative-stress mediated signals for full-blown reactions (Boldogh et al. 2005).

Naturally, the primary role of pollen NAD(P)H oxidases is not the disturbance of normal function of the airways, then why do pollens need/have these enzymes? During the plant fertilization process, a pollen tube grows from the pollens that land on the stigma of the receptive flower. This process demands high energy and requires rapid oxygen uptake, and it is regulated by Rac homologs and Ca^{2+} signaling (Kost et al. 1999; Pierson et al. 1996; Tadege and Kuhlemeier 1997). It was reported by Foreman et al. that ROS are required for the elongation of plant root hairs growth. It is turned out that ROS

produced by plant NAD(P)H oxidases act on hyperpolarisation-activated Ca^{2+} channels necessary for the formation of the tip-high Ca^{2+} gradient, a characteristic of all tip-growing cells, including pollen tubes, fungal hyphae and root hairs (Foreman et al. 2003). Later it was reported that NADPH oscillates in pollen tubes which is in correlation with tip growth (Cardenas et al. 2006) and the ROS produced by the enzyme require to pollen tube growth (Potocky et al. 2007).

2.4 Static magnetic field

The research field investigating the impact of static magnetic field (SMF) on biological systems has been rapidly growing for many years. However, the precise biological effects caused by the exposure are not well known. The 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 the applied SMF. Generally, based on the strength of SMF we differ weak-intensity SMF in the microtesla (μT) range, including the geomagnetic field (varying from 20 to 90 μT with a typical value around 50 μT), moderate-intensity SMF in the millitesla (mT) range, and strong-intensity SMF in the tesla (T) range, including ultrastrong-intensity SMF (more than 5 T) such as magnetic resonance imaging (MRI) device (Okano 2008).

Several studies have shown that magnetic fields influence a large variety of cellular functions; however, the mechanisms of interactions between SMF and living cells remain unclear. One of the potential mechanisms by which the biological systems respond to various external magnetic fields is acting through electronic interactions, i.e., radical pair mechanisms. SMF may directly interact with free radicals affected by their membrane processes and related with the physiological functions. Another possible mechanism might be related to the changes in antioxidant/oxidant status of the organism (Traikov et al. 2009). It is assumed that SMF can change the lifetime of radical pairs. SMF can influence the spin of electrons in free radicals, which may lead to changes in chemical reaction kinetics and possibly alters cellular function (Brocklehurst and McLauchlan 1996).

It was demonstrated that 1.5 T SMF induced positive effect by decreasing oxidative stress in men following short-term exposure in the MRI apparatus. The total antioxidant capacity of the individuals showed a significant increase in post-exposures when compared with pre-exposures to the SMF. The oxidative stress index and total

oxidant status showed a significant decrease in post-exposures when compared with pre-exposures to the 1.5 T magnetic field (Sirmatel et al. 2007). In a mouse study, SMF exposure, began 3.5 h after induction of acute inflammation by carrageenan, markedly decreased the level of malondialdehyde (MDA) a marker of oxidative stress and SOD in the blood (Traikov et al. 2009).

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 (Ghodbane et al. 2013). Further, it has been reported that low-intensity magnetic fields increased the cellular lifespan of ROS. SMF can also promote a Fenton-like reaction with the formation of hydroxyl radicals, which damage lipids, proteins, DNA and in turn calcium homeostasis (Lai and Singh 2004).

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 carefully examined the impact of SMF on the antinociceptive effect observed in mice testing more than 20 different devices containing permanent magnets. It was determinated that the stronger the SMF or the higher the inhomogeneous level of SMF is, the more expressed effect SMF shows. Furthermore, magnets on both sides of the treated area and the coupling their opposite poles also contribute to the increased antinociceptive effect of SMF. A typical surface field map of the inhomogeneous SMF at a distance of 10 mm from the surface of the magnetic device which showed the best results is presented in Figure 5 (Laszlo et al. 2007).

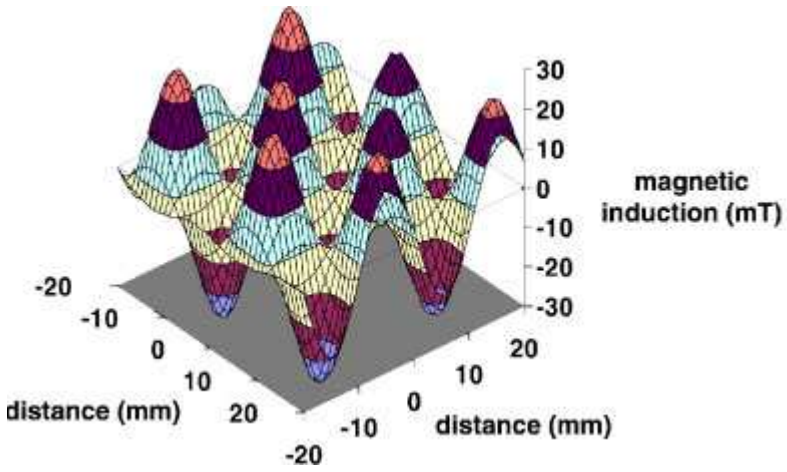

Figure 5. Field map of inhomogeneous SMF. *A typical field scan of SMF at a distance of 10 mm from the magnets' surface for the case of NdFeB N50 cylindrical magnets sitting one next to another with alternating polarity. The representative scanned area was 41x41 mm in the center of the matrices. Here the contribution of the asymmetrical magnetic induction (flux density) of the matrix edges could be neglected (Laszlo et al. 2007).*

Based on these previous data and the possibility that we are able to generate a potentially effective inhomogeneous SMF on biological systems, 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 impacts of SMF-exposure on pollen-induced allergic inflammation.

2.5 Aims of the studies

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

- To study whether oxidative stress induced by exposure to pollen grains contribute 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 Ag-dependent adaptive immune responses;

- To check whether inhomogeneous static magnetic field 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. Materials and Methods

3.1 Materials

Sterile plastic plates were purchased from TPP (Trasadingen, Switzerland). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

3.2 Studies on pollen-exposed human monocyte-derived DCs

3.2.1 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). 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. Human peripheral blood mononuclear cells (PBMCs) were separated from buffy coats of healthy blood donors 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), followed by washing with phosphate buffered saline (PBS)-0.5% bovine serum albumine (BSA)-2mM ethylenediaminetetraacetic acid (EDTA). To generate immature DCs (iDCs), cells (96-99% of them displayed the monocyte phenotype) 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. On day 5 the individual DC cultures were phenotypically characterized and they were found to be DC-SIGN/CD209$^+$, CD11c$^+$, HLA-DR$^+$ and CD14^{low} and the ratio of CD1a$^+$ DCs varied among individuals between 25% and 96% as determined by flow cytometry.

3.2.2 Treatments 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 preliminary experiments, different concentrations of RWPs (ranging from 5-400 µg/ml) were tested for their ability to increase the intracellular ROS levels in DCs and a plateau was reached at the concentration of 100 µg/ml resulted in the 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). To study the effects of oxidative stress on DC function, antioxidant (10 mM N-tert-butyl-α phenylnitrone; PBN) was added to the cell cultures 1 h prior the pollen exposure. 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.

3.2.3 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 iDCs 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. Relative ROS levels are given in arbitrary units of mean intensity of DCF fluorescence.

3.2.4 Analysis of cell surface receptor expressions by flow cytometry

Phenotypic characterization of DCs was performed 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).

3.2.5 T cell proliferation assay

The fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes), at the concentration of 0.5 µM, was used for the analysis of T cell proliferation. The CFSE-labeled T cells can be used for the proliferation assay due to the progressive halving of fluorescence within daughter cells following each cell division. Naïve $CD4^+$ T cells were purified from PBMCs by negative selection using the naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. T cell preparations contained 96% to 98% $CD4^+$ cells, of which 90% to 95% were $CD45RA^{high}$ and 1.8 to 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 CFSE-labeled allogeneic, naïve $CD4^+$ T cells in 96-well cell culture plates for 4 d in the presence of 0.5 µg/ml purified anti-human CD3 (BD Pharmingen) at a DC/T ratio of 1:20. As a control, untreated DCs were used. T cells stimulated with 10 µg/ml phytohaemagglutinin (PHA) were used as positive control. Fluorescence intensities were measured by FACSCalibur flow cytometer and data were analyzed by WinMDI software.

3.2.6 T cell activation by autologous DCs

To investigate the cytokine secretion profile of T lymphocytes primed with pollen-exposed DCs, monocytes as well as naïve $CD4^+$ T cells were isolated from buffy coats obtained from 3 ragweed allergic and 3 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 by means of screening for total and specific IgE using enzyme linked immunosorbent assay (ELISA) (Adaltis Italia S.p.A., Bologna, Italy). Sera containing less than 0.36 kU/L ragweed specific IgE and low level of total IgE (< 20 IU/mL) were classified as "non-allergic" and those with elevated ragweed specific IgE levels (0.72-17.99 kU/L) were regarded as "ragweed allergic" samples. Monocytes and naïve $CD4^+$ T cells were isolated as described above. 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 (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 stored at -20°C until cytokine measurements.

3.2.7 Determination of cytokine secretion by DCs and T cells

Set of cytokines secreted by DCs was determined by using ELISA kits specific for human IL-6, IL-8, IL-10, IL-12(p70), TNF-α, IL-1β (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).

3.2.8 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. Intracellular Foxp3 and IL-10 staining was performed by using eBioscience reagents according to the manufacturer's protocol. 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).

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

3.3.1 Inhomogeneous SMF exposure system

The inhomogeneous SMF was generated with an exposure system identical to the one which has previously been described in detail, carefully tested and optimized for small experimental animals by László et al. /#1 in (Laszlo et al. 2007)/. The device consisted of 2 ferrous matrices (size 170x140 mm) containing 10x10 mm cylindrical neodymium iron boron (NdFeB) N35 grade magnets (B_r=1.20 T). The lateral periodicity was 10 mm. 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. The magnetic device with the Plexiglas animal cage is illustrated on Figure 6.

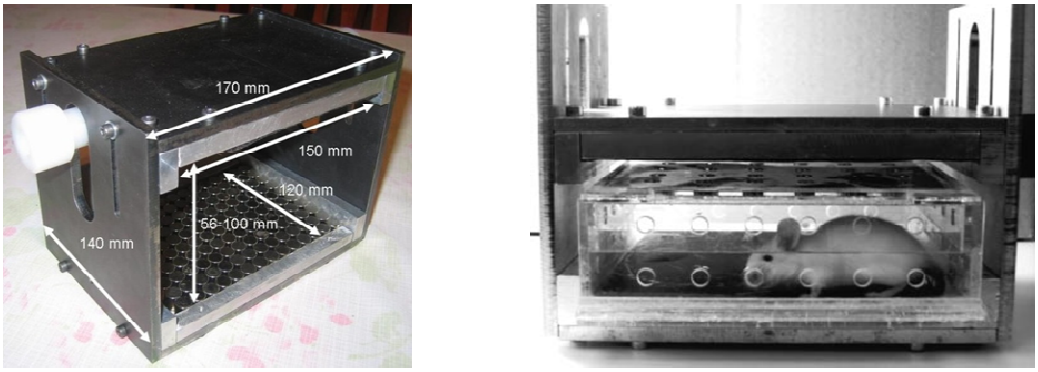

Figure 6. Device for induction of static magnetic field. *The device contains two magnet holding matrices, one below and another on top of the plastic animal cage. In the two matrices the poles of the individual magnets facing each other are oriented with opposite polarity allowing field lines to cross the cage, thus the magnetic field is vertically directed (Gyires et al. 2008; Laszlo et al. 2010).*

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.

For the skin prick tests a single magnetic matrix of the above exposure system was used /case #3 in (Laszlo et al. 2007)/. Along the shorter sides (on the contact sites) 2 spacers with soft surface coverage were fixed fulfilling 2 tasks: (i) ease holding the 875 g device on the forearm for 15 min, and (ii) provide a uniform distance of 3 mm between the magnetic (or sham) surface and the skin. On one forearm the magnetic matrix, on the other a sham matrix of identical looks and weight was used simultaneously. A random list

prepared prior to the test decided which device was applied on which forearm. This list was neither revealed to the volunteer, nor to the physician. No metallic object whatsoever was allowed at arm's reach before and during the test.

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 neighboring local extremes were 39.25 and 0.22 T/m at 3 and 15 mm from the surfaces of matrices, respectively /case #1 in (Laszlo et al. 2007)/. 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 (Laszlo et al. 2007). Components of the magnetic induction perpendicular to the magnets' symmetry axis were one order of magnitude smaller than the parallel ones and were regarded as stray field components. The dosimetric measurements were carried out by a gaussmeter (Lake Shore Cryotronics, Model 420, Westerville, OH, USA).

3.3.2 Experimental conditions during SMF exposure

As lighting is an important issue for both the *in vitro* tests and the *in vivo* mouse experiments, we took special care of balancing the SMF- and the sham-exposed situations. In short, the walls of both SMF- and sham-exposed cell culture plates were transparent to visible light, but the exposures were carried out in total darkness. The Plexiglas animal cages were covered with an opaque material on top and bottom and on 2 opposite sides. The cages in the SMF-exposure chamber were identically illuminated as under sham-exposure: through the front and back sides of the cages. Illumination was provided by fluorescent lights (Model L58W/640, Osram, Munich, Germany) from above during the experiments. The fluorescent lamps generated a basically horizontal scattered light in the shaded area of the Plexiglas cages with intensity between 6 and 15 mW/m^2 as previously described (Kiss et al. 2013). The applied light sources emit light with several wavelength peaks between 403 and 710 nm. The calculated integral light intensity maximum corresponds to $3.9x10^{16}$ $photons/s/m^2$, which is below the intensity threshold for altering behavioral responses in mice in SMF-shielded environments (Prato et al. 2009). The illumination conditions inside the cage were basically independent of the location of the mouse within the cage. Horizontal light gradients did not occur between front and back sides. We carried out the *in vivo* animal experiments in the same period of time of the day, between 8 and 12 a.m. being aware that rodents are subject to the circadian cycle in almost all areas of their life (Cain et al. 2004). Due to the closed design

of the Plexiglas animal cages, ventilation through the cages was restricted to the front-back direction for both SMF- and sham-exposed animals. Temperature and relative humidity were kept constant within prescribed limits during the experiments. In the *in vitro* tests, all cell culture plates were covered and incubated at 22°C during SMF- and sham-exposures.

Experiments were conducted in Debrecen, Hungary (47˚31′ N, 21˚38′ E). The background SMF in the lab was the geomagnetic field, the horizontal components of this field needed to be taken into account (total magnetic induction of 21 138.75 nT, grown to 21 160.79 nT during the the time period of the studies (NOAA)). Although the geomagnetic field itself is known to have the capacity to affect living organisms (e.g., migrating animals), in the present studies we only looked for the differences in the biological response to exposure to an artificial external SMF of at least four orders of magnitude stronger than that of Earth. This stronger SMF was simply superimposed for the magnetic background in case of SMF-exposed objects.

3.3.3 Animal experiments

3.3.3.1 Animals, sensitization, challenge and SMF exposure

Experiments were performed according to the Helsinki Declaration, European Union regulations and adhered to the guidelines of the Committee for Research and Ethical Issues of IASP. All animal study protocols were approved by the Animal Care and Protection Committee at the University of Debrecen (#7/2011/DE MAB). Animals were maintained in the pathogen-free animal facility of the University of Debrecen.

Female 8-week-old Balb/c mice (Charles River, Wilmington, MA, USA) were used for these studies. Animals were randomly divided into experimental groups. 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. Identical SMF-exposure was shown not to cause any change in the anxiety and locomotive behavior of mice (Laszlo and Gyires 2009).

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) as

previously described (Boldogh et al. 2005). On day 11, mice were challenged intranasally with 100 µg RWPE dissolved in 60 µl of PBS (PAA Laboratories, Pasching, Austria) or same volumes of PBS as a vehicle. On day 14, mice were euthanized by intraperitoneal injection of 250 µl xylazine (2 mg/ml) (CP-Pharma, Burgdorf, Germany) and ketamine (18 mg/ml) (Richter Gedeon Plc., Budapest, Hungary) and allergic inflammation was evaluated. To test whether SMF-exposure had an effect on allergic airway responses of the treated animals and to determine the optimal timing of exposure and the size of animal groups required for reasonable statistical considerations we first performed a pilot study.

3.3.3.2 Animal groups in the pilot study

- *Group A* Animals (*n*=5) were exposed to sham field (no SMF) for 30 min a day from day 0 through day 13 (sham treatment on day 11 was performed immediately after intranasal RWPE challenge)
- *Group B* Animals (*n*=5) were exposed to SMF for 30 min a day from day 0 through day 11 (last treatment was performed 6 h prior to intranasal RWPE challenge)
- *Group C* Animals (*n*=5) were exposed to SMF for 30 min a day from day 11 through day 13 (treatment on day 11 was performed immediately after intranasal RWPE challenge)
- *Group D* Animals (*n*=5) were exposed to SMF for 30 min a day from day 0 through day 13 (treatment on day 11 was performed immediately after intranasal RWPE challenge)
- *Group E* Animals (*n*=3) were exposed to SMF for 30 min a day from day 0 through 13 (treatment on day 11 was performed immediately after intranasal PBS challenge)

3.3.3.3 Animal groups in the full test

- Group I Animals (*n*=19) were exposed to sham field (no SMF) for 30 min a day from day 11 through day 13 (treatment on day 11 was performed immediately after intranasal RWPE challenge)
- Group II Animals (*n*=21) were exposed to SMF for 30 min only on day 11 immediately after intranasal RWPE challenge
- Group III Animals (*n*=21) were exposed to SMF for 30 min a day from day 11 through day 13 (treatment on day 11 was performed at 6 h after intranasal RWPE challenge)
- Group IV Animals (*n*=8) were exposed to SMF for 60 min a day from day 11 through day 13 (treatment on day 11 was performed at 6 h after intranasal RWPE challenge)
- Group V Animals (*n*=8) were exposed to SMF for 30 min a day from day 11 through day 13 (treatment on day 11 was performed at 6 h after intranasal PBS challenge)

The experimental protocols are summarized in Figure 7.

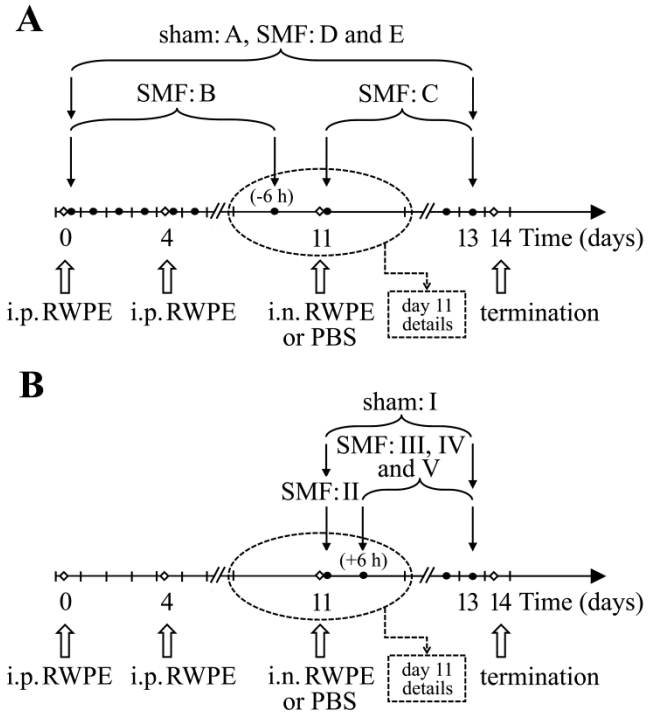

Figure 7. Experimental protocols for the pilot study (a) and the full test (b). *Mice were divided into 5 groups both in the pilot study (A-E) and in the full test (I-V). Black dots indicate the dates of static magnetic field (SMF)-exposures and open squares represent the dates of intraperitoneal (i.p.) and intranasal (i.n.) administration of ragweed pollen extract (RWPE) or PBS or termination of the experiment, respectively.*

3.3.3.4 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. To collect BALF, animals were euthanized and their tracheas were cannulated. Lavage was performed with 2 aliquots of 0.7 ml of ice cold PBS (pH 7.3). The BALF samples were centrifuged (400g for 10 min at 4°C), the supernatants were removed and stored at -80°C for further analysis. 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, the lungs were fixed following BALF collection by inflating with formalin. 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.

3.3.3.5 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. Plates were blocked with 2% BSA in PBS for 1 h and incubated with 50 µl (1:10,000 dilutions) of biotin-conjugated mouse monoclonal MUC5AC antibody (Lab Vision, Fremont, CA, USA). After 60 min the plates were washed with PBS-BSA and further incubated with streptavidin-horseradish peroxidase goat anti-mouse IgG conjugate (1:10,000) for 1 h. Plates were washed (twice with PBS-BSA) and were incubated with peroxidase substrate (3,3',5,5'-tetramethylbenzidine) to obtain the colorimetric product, which was quantified at 450 nm. Results were expressed as endpoint titers (Frey et al. 1998).

3.3.3.6 Assay for total antioxidant potential

To measure total antioxidant capacity of the airways, naïve mice (n=12) were intranasally challenged with RWPE (100 µg dissolved in 60 µl PBS) or with identical volumes of PBS immediately preceding the exposure to SMF or sham field for 30 min. Bronchoalveolar lavage was performed 15 min after treatment as described above. The BALF samples were centrifuged (400g for 10 min at 4°C) 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). A standard of known 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, included in the kit) concentration was used to create a calibration curve (R^2=0.999) and the results of the assay were expressed as nmol/µL Trolox equivalents.

3.3.4 In vitro studies

3.3.4.1 Cell cultures

The A549 human bronchial epithelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), penicillin (100 U/mL), and streptomycin (100 µg/mL).

3.3.4.2 Assessment of intracellular ROS levels in cultured epithelial cells

A549 cells grown to 70% confluence 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 100 µM NADPH or RWPE (100 µg/ml) plus NADPH (100 µM). Immediately following this treatment the cell cultures were sham- or SMF-exposed for 30 min either at lower or upper position (see in Section 3.3.1). Changes in DCF fluorescence intensity were assessed in a Synergy HT micro plate reader at 488 nm excitation and 530 nm emission.

3.3.4.3 Measurement of RWPE-generated ROS under cell-free conditions

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. Plates were exposed to sham field or to SMF either at lower or upper position for 30 min and changes in the DCF fluorescence intensity were determined using a Synergy HT micro plate reader at 485 nm excitation and 528 nm emission.

3.3.5 Human study

3.3.5.1 Participants in the skin prick test, ethics

The study population consisted of 62 volunteers (21 males and 41 females, age between 22 and 50 years). Exclusion criteria of the study were: pregnancy or lactating, using medication for allergies, or abnormal spirometry test results. 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 (Debrecen, Hungary, # DE OEC RKEB/IKEB 3854-2013) and conducted at the Department of Pulmonology of the University of Debrecen under the supervision of a specialist. The tests were carried out in April-May 2013. No regulation for the use of permanent magnetic devices is available in the European Union which magnetic induction is below 8 T. For potential risks of SMF-exposure, see the report of the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR 2009). Colbert and co-workers proposed a standardization of the

description of clinical study reports including SMF (Colbert et al. 2009). Table 1. contains our corresponding data.

Target tissue	inner forearm skin following provoked skin allergy test
Site of magnet application	both forearms: simultaneous sham-exposure on one and SMF-exposure on the other
Distance of magnet surface from target tissue(s)	3 mm
Magnetic field induction	B_r=1.20 T (remanent induction), 192.28±0.1 mT (peak-to-peak magnetic induction, averaged for all neighbors) by 18.89 T/m lateral gradient (lateral magnetic induction gradient of the main induction component, averaged for all neighbors) at 3 mm from a cylindrical magnet in the isocenter of the matrix along its axis
Material composition of permanent magnet	N35 grade neodymium-iron-boron (NdFeB)
Magnet dimensions	rectangular matrix (140x100 mm) containing 140 pieces of 10x10 mm cylindrical magnets
Magnet polar configuration	neighboring magnets are placed with alternating poles (checkerboard configuration)
Magnet support device	ferrous plate above the magnets, spacers with soft coverage on the contact sites
Frequency of magnet application	single session, continuous exposure
Duration of magnet application	15 min

Table 1. Ten essential dosing parameters for the present study *(as suggested by Colbert et al. 2009).*

3.3.5.2 Skin prick test on human volunteers

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). In order to minimize the variability of the results (the volume of administered test samples and the depth of scrapes) Multi-test II applicators (Lincoln Diagnostics, Decatur, IL, USA) were used. 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.

Finally, 12 test results out of 62 were excluded from the statistical assessment. The reason was one of the followings: (i) wheal of 3 mm or bigger in diameter response to the negative control indicating severe dermatographism (n=4); (ii) nonidentical responses to the negative control on the left and right forearms (n=8). The remaining sample population contained 15 males and 35 females of average age 30.9 years.

3.4 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. All analyses were performed by using SPSS Statistics software, version 17.0.

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. Statistical analyses were made using Excel (Microsoft) and XLSTAT v. 7.5 softwares (Addinsoft). Differences were considered to be statistically significant at $p < 0.05$. We also defined the effect in percent meaning M=1-(value in one group)/(value in other group). By such a definition, the effect can be negative and can exceed 100% in absolute value. In both studies, the normality of the distribution of measured data was checked by using Kolmogorov-Smirnov tests in all measurements.

4. Results

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

Airway DCs, predominantly located underneath the epithelial basement membrane, extend their long processes between epithelial cells into the airway lumen (Takano et al. 2005); thus they are able to come into direct contact with the inhaled pollen grains. We used human monocyte-derived DCs to investigate the consequences of pollen exposure on dendritic cell function. Because previous studies have indicated that RWPs possess NAD(P)H oxidase activity, which generates reactive oxygen radicals (Bacsi et al. 2005; Boldogh et al. 2005), we presumed that exposure to pollen grains would increase the intracellular level of ROS in cultured monocyte-derived DCs.

To test this hypothesis, iDCs were loaded with a redox-sensitive fluorescent dye, H_2DCF-DA and RWPs were added to the cell culture. Pollen exposure rapidly induced a 5.9 $\pm$ 2.1 -fold increase of intracellular DCF fluorescence, which could be prevented by heat treatment of the pollen grains (Figure 8). Pretreatment of RWPs with DPI, a NAD(P)H oxidase inhibitor, also significantly decreased the elevation of intracellular ROS levels (Figure 8). 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 (Thomas et al. 1996). The capacity of PBN molecules to neutralize free radicals provides functional activities similar to those of antioxidants (Butterfield et al. 1998). 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 (Figure 8). 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.

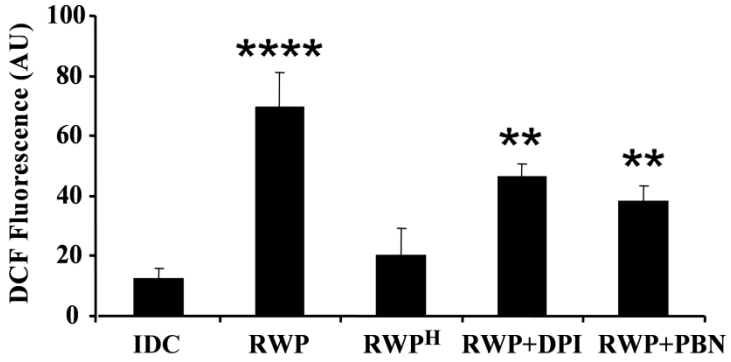

Figure 8. Exposure to ragweed pollen grains increases the intracellular ROS levels in cultured monocyte-derived DCs. *Cells were loaded with H_2DCF-DA and after removing excess probe, treated as indicated. Changes in DCF fluorescence intensity were detected by means of fluorimetry. Data are presented as means ± SEM of four independent experiments. ** $p < 0.01$; **** $p < 0.0001$ vs IDC control. AU, arbitrary unit; DCF, dichlorofluorescein; IDC, untreated DCs; RWP, DCs exposed to ragweed pollen grains; RWP^H, DCs exposed to heat-treated ragweed pollen grains; DPI, diphenyleneiodonium; PBN, N-tert-butyl-α phenylnitrone.*

4.2 Pollen-induced oxidative stress influences the maturation state of DCs

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

It was shown previously that ROS either directly (Verhasselt et al. 1998) or via oxidatively modified glycoproteins (Buttari et al. 2005) (Buttari et al. 2005) 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, IL-1β) secretion.

After 24 h of the pollen exposure, we found that 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-treated DCs than in those of unstimulated cells (Figure 9A-C). However, treatment of DCs with pollen grains did not induce the secretion of IL-1β 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 (Figure 9A-C). Pretreatment with PBN alone did not affect the chemokine and cytokine release from DCs (data not shown). Exposure to heat treated pollen grains, in which the activity of the intrinsic NAD(P)H oxidases was eliminated (Bacsi et al. 2005), led to significantly lower mediator release by DCs compared to those treated with intact pollen grains. However, the cytokine and chemokine production by DCs exposed to heat treated pollen grains was higher than the basal level. (Figure 9A-C).

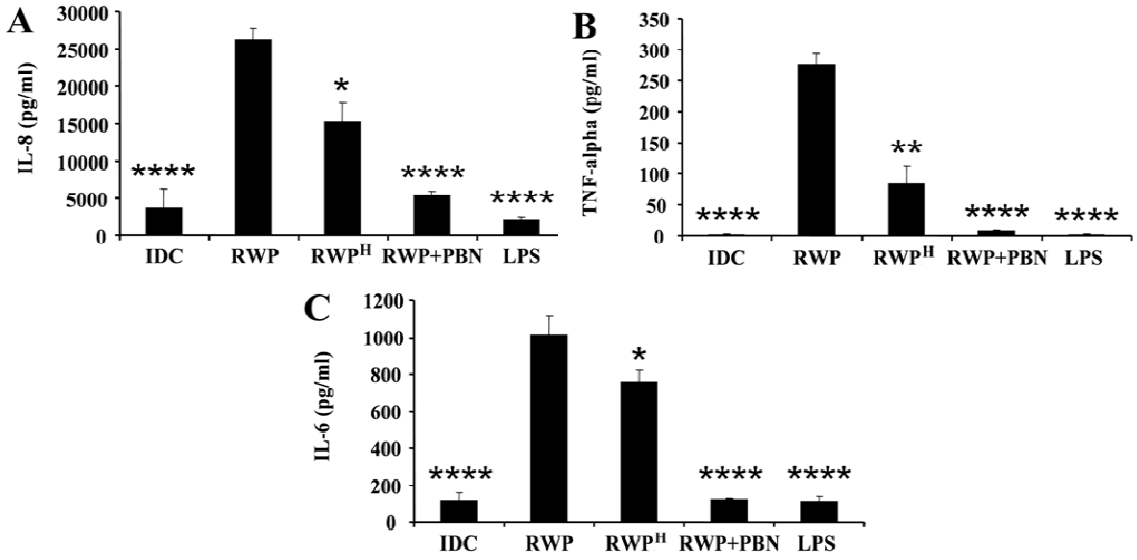

Figure 9. Effect of pollen exposure-induced oxidative stress on the chemokine and cytokine producing capacity of DCs. *Levels of IL-8 (A), TNF-α (B) and IL-6 (C) in the culture supernatants of pollen-treated DCs were determined 24 h after the exposure by means of ELISA. LPS contamination of RWP sample was determined and equivalent amount of LPS from E. coli (16 pg/ml) was used as control. Data are presented as means ± SEM of four to five independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs pollen-treated DCs. IDC, untreated DCs; RWP, DCs exposed to ragweed pollen grains; RWP^H, DCs exposed to heat-treated ragweed pollen grains; PBN, N-tert-butyl-α phenylnitrone; LPS, LPS-treated DCs.*

It has previously been reported that LPS can also induce oxidative stress and trigger the secretion of various cytokines from monocyte-derived DCs (Yamada et al. 2006). 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 (16 pg/ml) of LPS from *Escherichia coli* as a control. Treatment of iDCs with this amount of LPS could not induce IL-6, IL-8 or TNF-α release from the cells (Figure 9A-C).

4.2.2 Oxidative stress triggered by pollen exposure contributes to the phenotypic maturation of DCs

During delivery of antigen to local lymphoid tissues, phenotypic and functional changes of DCs are needed to prime naïve 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, iDCs were also treated with LPS (16 pg/ml). The expression of costimulatory molecules (CD40, CD80 and CD86), CD83, a specific maturation marker, and the antigen presenting molecule HLA-DR was analyzed by flow cytometry.

As shown in Figure 10, treatment of iDCs with pollen grains resulted in a slight increase in the expression of CD40 (Relative Fluorescence Intensity [RFI] from 20.80 to 25.83); while 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) 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; however pre-treatment with PBN alone did not modify the phenotypic characteristics of DCs (data not shown). 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.

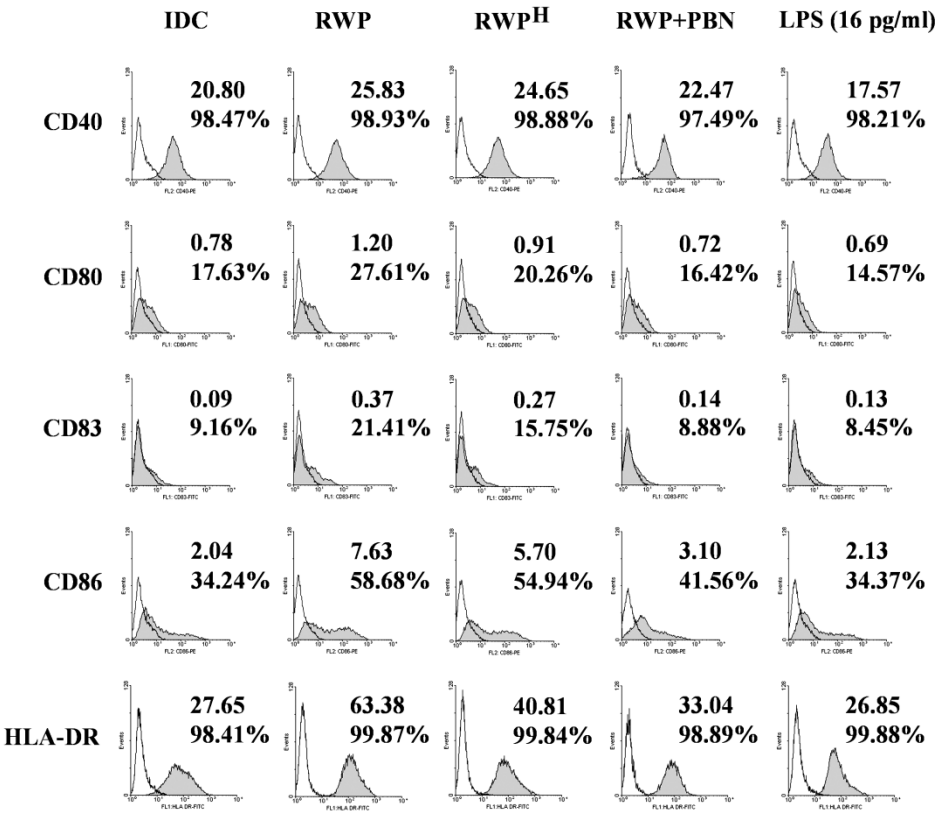

Figure 10. Pollen exposure-induced oxidative stress contributes to phenotypic maturation of DCs. *Immature DCs were treated with RWP for 24 h and the expression of HLA-DR, costimulatory molecules or maturation marker was analyzed by flow cytometry. Unfilled histograms indicate isotype controls. Numbers show the relative fluorescence intensity, RFI (upper) and the percentage of positive cells (lower). A representative result of six independent experiments is demonstrated. IDC, untreated DCs; RWP, DCs exposed to ragweed pollen grains; RWP^{H}, DCs exposed to heat-treated ragweed pollen grains; PBN, N-tert-butyl-α phenylnitrone; LPS, LPS-treated DCs.*

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.

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. The responsiveness of CFSE-labeled naïve $CD4^+$ T lymphocytes to alloantigens presented by monocyte-derived DCs was analyzed by flow cytometry after 4 d of stimulation. A faster dilution of the fluorescent dye in dividing T cells indicated that the pollen-treated DCs had a strong stimulatory effect on T cell proliferation compared to iDCs (80.8% versus 56.6%, Figure 11). To investigate whether pollen exposure-induced oxidative stress affects the allostimulation, heat-treated RWPs and PBN were applied. We found that both treatments (71.6% for $RWPE^H$ and 60.5% for PBN; Figure 12) 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%, Figure 11). As positive control, naïve $CD4^+$ 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.

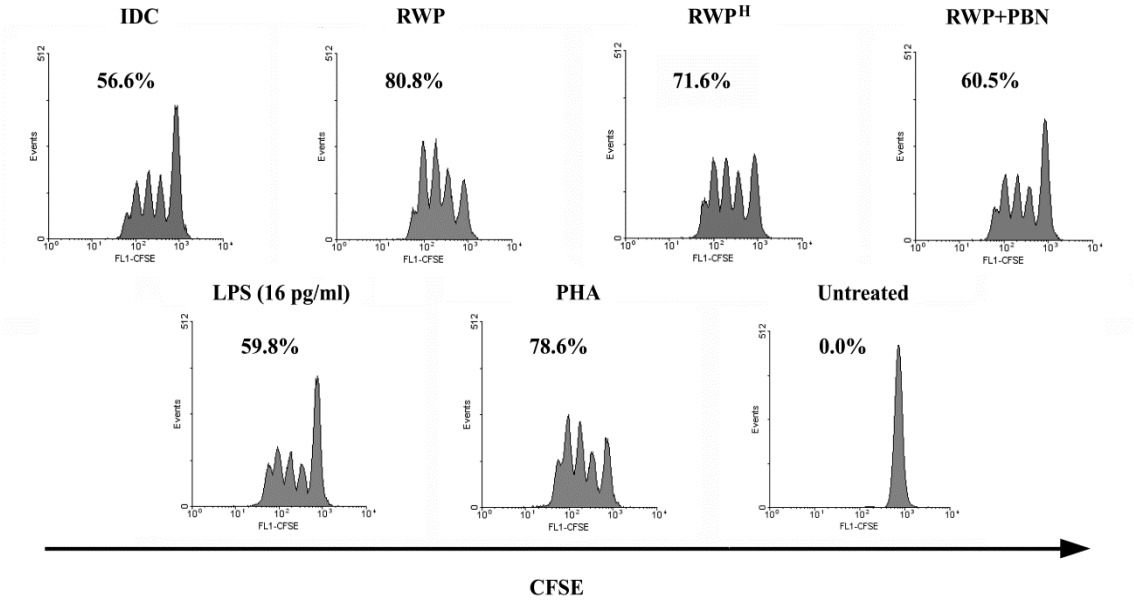

Figure 11. Increase in the intracellular ROS levels upon pollen exposure alters the T cell priming capacity of DCs. *Pollen-treated DCs were cocultured with CFSE-labeled allogeneic naïve $CD4^+$ T cells for 4 days and then fluorescence intensities were measured by flow cytometry. Numbers indicate the proportion of dividing T cells. Results are representative of four independent experiments. IDC, untreated DCs; RWP, DCs exposed to ragweed pollen grains; RWP^H, DCs exposed to heat-treated ragweed pollen grains; PBN, N-tert-butyl-α phenylnitrone; LPS, LPS-treated DCs; PHA, phytohaemagglutinin.*

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

Dendritic cells 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. 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 antioxidant (PBN) or treatment with heat-inactivated pollen grains had no significant effect on basal IL-12 release (Figure 12A). Pollen-treated DCs produced 22.2 ± 4.5 pg/ml of IL-10 and this level of the cytokine was significantly higher than that released by untreated DCs (Figure 12B). 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 (Figure 12B). In control experiments, IL-10 production of DCs was not affected by treatment with LPS (16 pg/ml).

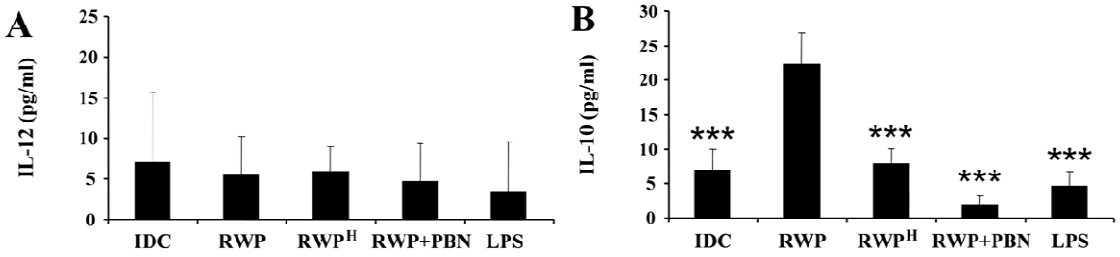

Figure 12. Effect of pollen exposure-induced oxidative stress on the production of IL-12 and IL-10 by DCs. *Levels of IL-12 (A) and IL-10 (B) in the culture supernatants of pollen-treated DCs were determined 24 h after the exposure by means of ELISA. Data are presented as means ± SEM of four to five independent experiments. *** $p < 0.001$ vs pollen-treated DCs. IDC, untreated DCs; RWP, DCs exposed to ragweed pollen grains; RWP^H, DCs exposed to heat-treated ragweed pollen grains; PBN, N-tert-butyl-α phenylnitrone; LPS, LPS-treated DCs.*

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 (37 $\pm$ 7.8 versus 12.3 $\pm$ 4.1 pg/ml, Figure 13A). 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 (Figure 13B,C). 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 to cells cocultured with untreated autologous DCs (Figure 13D). 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 (66.9 ± 15 versus 46.6 ± 9.6 pg/ml, Figure 13E). The same priming method did not stimulate increased release of IL-10 from T cells of non-allergic donors (Figure 13E). T cells from non-allergic subjects produced 19.5-fold higher amount of IFN-γ after priming with pollen-treated autologous DCs, compared to those from allergic donors (7771 ± 2681 versus 399 ± 149 pg/ml, Figure 13F). We were not able to detect the release of IL-7 from T cells in our experimental settings (data not shown). Because it has previously been reported that antioxidants influence T cell polarization capacity of DCs by generating Tregs through inhibition of endogenous oxidative pathways in DCs (Tan et al. 2005), we did not use PBN treatment as control in these series of experiments.

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. Flow cytometric analysis of cells stained for CD4, CD25 and intracellular expression of Foxp3 showed that isolated naïve T cells are contaminated by 0.85 ± 0.03% $CD4^+CD25^+Foxp3^+$ T cells (data not shown). Priming T cells with iDCs 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, $p < 0.001$ vs. priming with iDCs), while priming with heat-inactivated pollen-treated DCs further increased the rate of $CD25^+Foxp3^+$ T cells up to 3.61 ± 0.03% (n=3, $p < 0.001$ vs. priming with pollen-exposed DCs) (Figure 14A). Simultaneous staining for CD25, intracellular IL-10 and Foxp3 identified a low ratio of IL-10-producing cells (0.28 ± 0.04%, Figure 14B,C). Priming with pollen-treated DCs did not change the ratio of $IL\text{-}10^+$ T cells as compared to the untreated ones (0.27 ± 0.07%, n=3, p =0.417 /NS/). However, stimulation with heat-inactivated pollen-exposed DCs led to a 2.3-fold increase in the proportion of $IL\text{-}10^+$ T cells (0.625 ± 0.18%, n=3, $p < 0.001$ vs. priming with pollen-exposed DCs) (Figure 14B,C). 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 (Figure 14B,C).

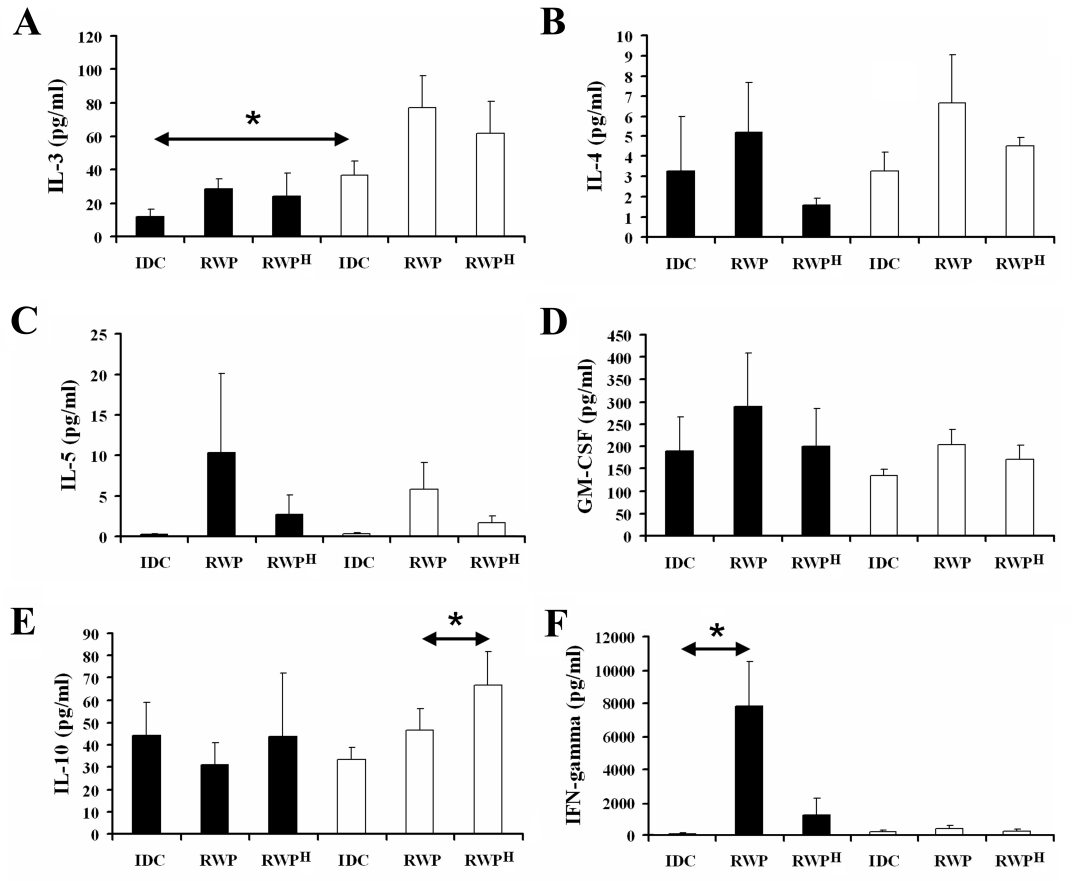

Figure 13. Pollen-induced oxidative stress influences the T cell polarizing capacity of DCs. *To investigate the cytokine secretion profile of T lymphocytes primed with pollen-exposed DCs, monocytes as well as naïve $CD4^+$ T cells were isolated from buffy coats obtained from 3 ragweed allergic (empty bars) and 3 non-allergic blood donors (black bars). Pollen-exposed DCs were cocultured with autologous naïve $CD4^+$ T cells for 4 days 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, while concentrations of IFN-γ (F) were measured by means of ELISA. Data are presented as means ± SEM of 3 independent experiments * $p < 0.05$. IDC, untreated DCs; RWP, DCs exposed to ragweed pollen grains; RWP^H, DCs exposed to heat-treated ragweed pollen grains.*

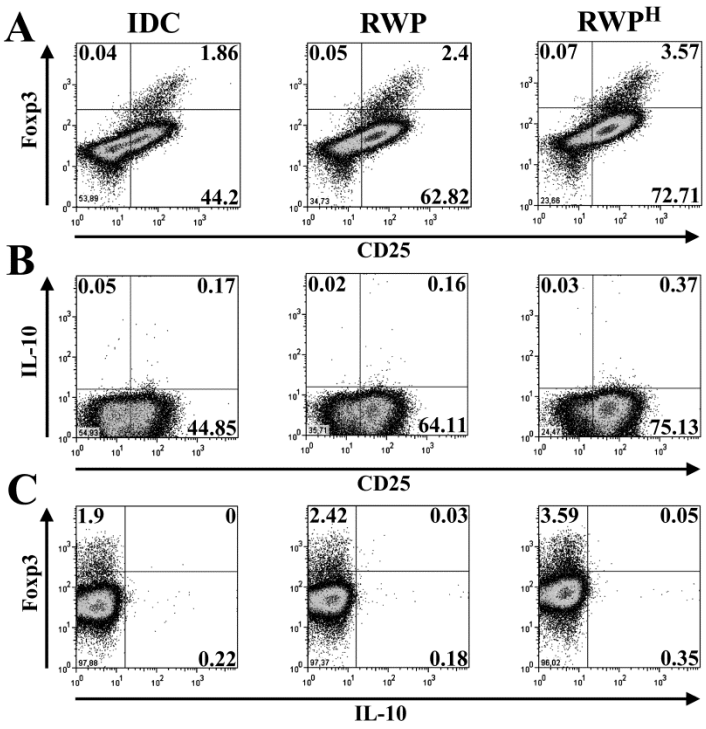

Figure 14. Characterization of IL-10 producing autologous T lymphocytes after coculturing with pollen-exposed DCs. *The intracellular IL-10 and Foxp3 staining was performed after anti-CD3 reactivation of the DC-primed T cells from ragweed allergic subjects adding monensin in the last 6 h of the stimulation. 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 antibodies. Results are representative of three independent experiments. IDC, untreated DCs; RWP, DCs exposed to ragweed pollen grains; RWP^H, DCs exposed to heat-treated ragweed pollen grains.*

Our data suggest that pollen-treated DCs - partly through pollen generated ROS - are able to induce the differentiation of naïve 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.

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 our pilot test mice were divided into 5 groups (A-E, see in Section 3.3.3.2 and Figure 7A) to investigate whether SMF-exposure has an effect on the sensitization or elicitation phase of RWPE-induced allergic reactions. Daily exposure to SMF during the 11-day long sensitization phase prior the intranasal RWPE challenge did not affect the accumulation of eosinophils in the BALF compared to sham-exposure (see Group B and A in Figure 15). 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 (days 11-13) after the intranasal challenge (see Group C in Figure 15). Daily exposure of RWPE-challenged mice to SMF during the 13-day long experimental period also decreased eosinophil counts in the BALF compartment as compared to RWPE-challenged, sham-exposed mice (see Group D and A in Figure 15). 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.

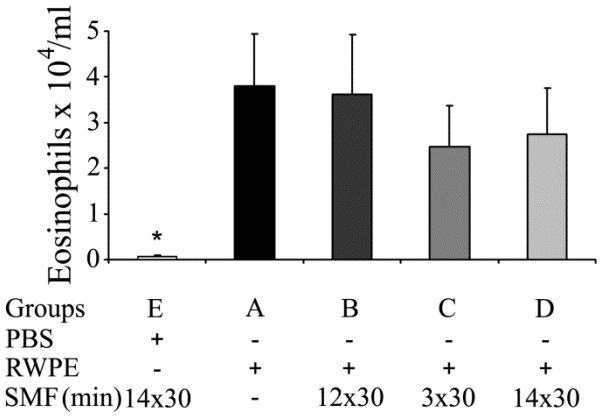

Figure 15. SMF-exposure does not affect the sensitization phase of the allergic responses. *RWPE- or PBS-challenged, sensitized mice were exposed to SMF or sham field for 30 min daily during sensitization (Group B) or elicitation (Group C) phase only or the whole period of the experiment (Groups A, D, E). Three days after challenge bronchoalveolar lavage (BAL) was performed and lavage samples were examined for eosinophil cell counts. Results are presented as means ± SEM. * $p < 0.05$ vs. RWPE challenged, sensitized mice exposed to sham field. PBS, phosphate buffered saline; RWPE, ragweed pollen extract; SMF, static magnetic field.*

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

In the full experimental series mice were divided into 5 groups (I-V), were sensitized with RWPE, challenged with RWPE or PBS, and exposed to SMF or sham field (see in Section 3.3.3.3 and Figure 7B). Exposure to SMF for a single 30 min time period immediately after the intranasal challenge on day 11 induced a moderate, but significant decrease in total cell counts in the BALF (Figure 17A) (M=14.90%, p=0.003), strongly and significantly decreased number of eosinophils in the BALF (Fig. 16B) (M=33.81%, p<0.001), and lowered the infiltration of inflammatory cells into the subepithelial area of the airways (Figure 16C) compared to RWPE-challenged, sham-exposed animals. A single SMF-exposure also decreased MUC5AC levels in BALF to some extent (Figure 17A) (M=14.63%, p=0.113) and also decreased epithelial cell metaplasia in the airways (Figure 17B) as compared to Group I. Significantly lower numbers of total cells (Figure 16A) (M=21.48%, p<0.001) and eosinophils (Figure 16B)

(*M*=26.50%, *p*<0.001) in the BALF together with decreased accumulation of inflammatory cells in the subepithelial area were also detected (Figure 16C) upon exposure to SMF for 30 min on 3 consecutive days (days 11-13) following RWPE challenge. This 3x30 min SMF-exposure significantly reduced MUC5AC levels (*M*=19.51%, *p*=0.035) in the BALF (Figure 17A) and markedly lessened epithelial cell metaplasia in the airways (Figure 17B) as compared to sham-exposed animals. Prolonged exposure to SMF (60 min a day) on 3 consecutive days (days 11-13) after RWPE challenge further decreased the total cell (*M*=26.67%, *p*<0.001) (Figure 16A) and eosinophil (*M*=57.70%, *p*<0.001) (Figure 16B) numbers in the BALF, and lowered inflammatory cell accumulation in the subepithelial regions of the airways as well (Figure 16C). Prolonged SMF-exposure was trendwise more effective to abate total (*M*=6.60%, *p*=0.207) and significantly more effective in lessening of eosinophil cell (*M*=42.45%, *p*<0.001) influx into the airways than exposures for 3x30 min (Figure 16B). Furthermore, the 3x60 min exposure to SMF was more effective in decreasing MUC5AC levels in the BALF (*M*=36.36%, *p*=0.019) (Figure 17A) and to lower epithelial cell metaplasia in the airways (Figure 17B) than the 3x30 min treatment. These results suggest that even a single 30 min exposure to SMF immediately after intranasal allergen challenge is able to decrease airway inflammation. In addition, increased doses of SMF-exposure either by increasing exposure time period (more days) or by extended duration of individual treatments (60 min instead of 30 min) can further decrease the severity of allergic inflammation in the lung.

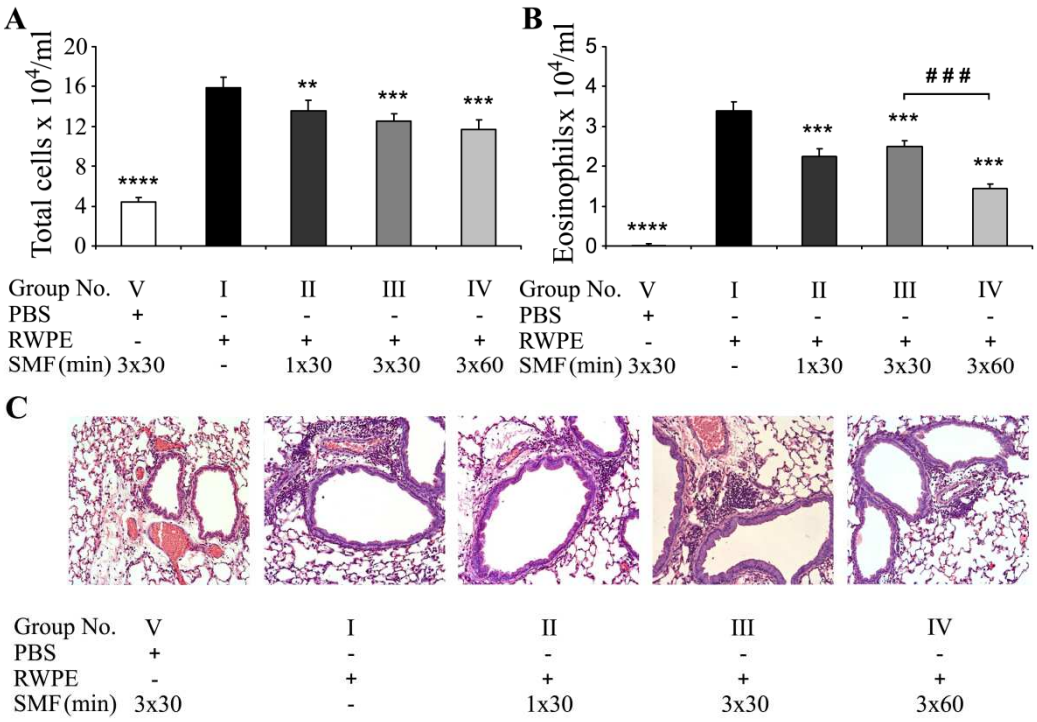

Figure 16. Exposure to SMF reduces RWPE-induced allergic airway inflammation. *Sensitized mice were challenged with PBS or RWPE and exposed to SMF or sham field. Three days after challenge BAL was performed and lavage samples were examined for (A) total and (B) eosinophil cell counts. (C) Hematoxylin and eosin staining of formalin-fixed lung sections. Original magnification 100x. Results are presented as means ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. RWPE challenged sensitized mice exposed to sham field. $^{###}$ $p < 0.001$, significant difference between Groups III and IV. PBS, phosphate buffered saline; RWPE, ragweed pollen extract; SMF, static magnetic field.*

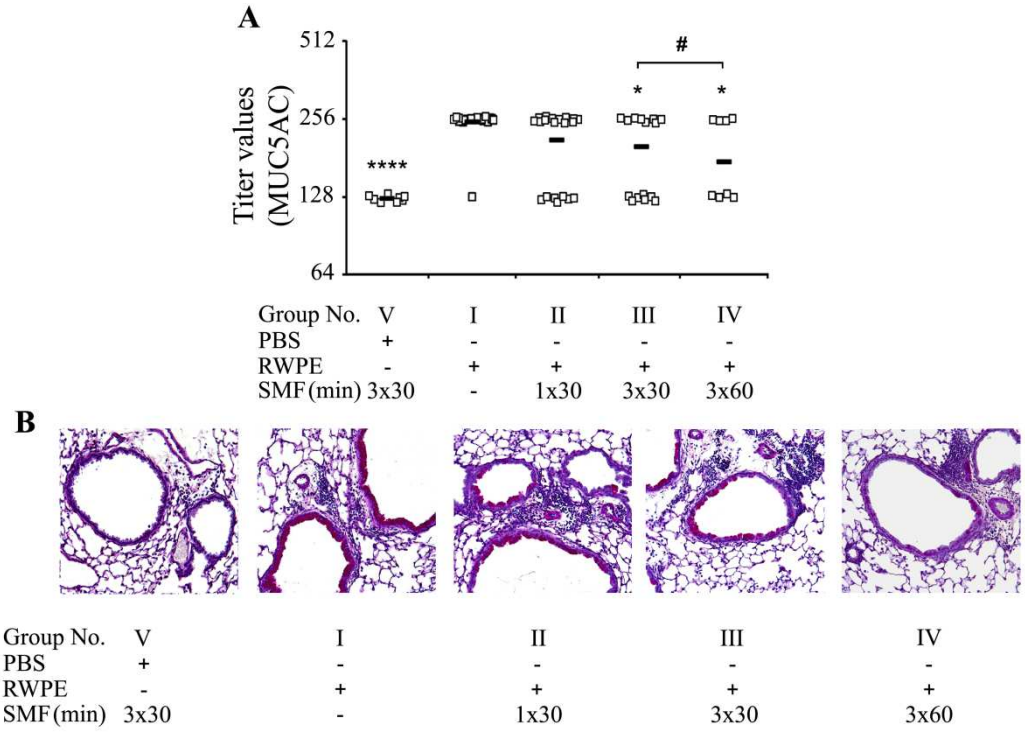

Figure 17. SMF-exposure decreases mucin levels and epithelial cell metaplasia in the airways of RWPE-challenged sensitized mice. *(A) MUC5AC levels in the BAL fluids of RWPE- or PBS-challenged sensitized mice exposed to SMF or sham field. MUC5AC levels were measured by means of ELISA and the results were expressed as endpoint titers (empty sqare) and means (-). * $p < 0.05$, **** $p < 0.0001$ vs. RWPE challenged, sensitized mice exposed to sham field, # $p < 0.05$, significant difference between Group III and IV. (B) Periodic acid-Schiff staining of formalin fixed lung sections. Original magnification 100x. PBS, phosphate buffered saline; RWPE, ragweed pollen extract; SMF, static magnetic field.*

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. In accordance with our previous work (Boldogh et al. 2005), sham-exposed RWPE converted the redox-sensitive H_2DCF-DA into DCF leading to 75 times higher fluorescence intensity (p<0.001) than that of PBS control (Figure 18). However, exposure to SMF for 30 min

either at lower or upper position (see in Section 3.3.1) did not alter DCF fluorescence signals induced by RWPE (Figure 18).

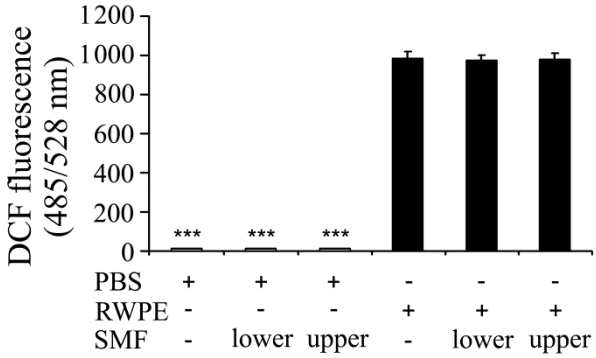

Figure 18. SMF-exposure does not alter the generation of ROS by RWPE in cell-free conditions. *PBS (empty bars) or RWPE (black bars) solutions containing redox-sensitive H_2DCF-DA were exposed to SMF or sham field for 30 min at lower or upper position (see Section 3.3.1). Changes in DCF fluorescence intensity were detected by means of fluorimetry. *** $p < 0.001$ vs. RWPE exposed to sham field. PBS, phosphate buffered saline; RWPE, ragweed pollen extract; SMF, static magnetic field.*

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. A549 cells loaded with H_2DCF-DA were treated with PBS or RWPE and immediately following this treatment the cell cultures were sham- or SMF-exposed for 30 min at lower or upper position (see in Section 3.3.1). Addition of RWPE to A549 cells induced a 3.6-fold increase (p<0.001) in intracellular DCF fluorescence signals compared to PBS treatment (Figure 20). The increase in intracellular ROS levels could significantly be diminished (M_{max}=20.57% at 135 min, p=0.002), when the cells were exposed to SMF at the lower position and also, to a smaller extent, when they were in the upper position (M_{max}=9.34% at 135 min, p=0.034) (Figure 19). Exposure to SMF, either at lower or upper position, did not cause significant changes in levels of intrinsic ROS in PBS-treated cells (Figure 19).

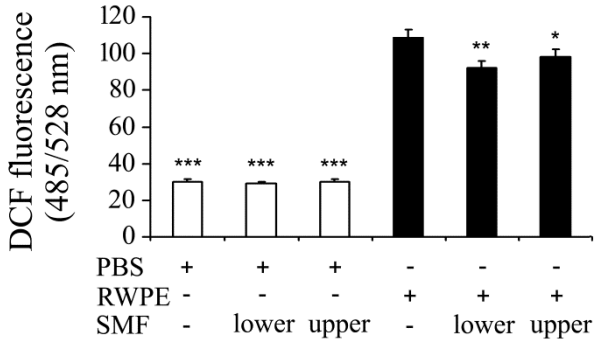

Figure 19. SMF-exposure diminishes the increase in intracellular ROS levels in RWPE-treated epithelial cells. *A549 cells loaded with H_2DCF-DA were treated with PBS (empty bars) or RWPE (black bars) and immediately after the treatment they were exposed to SMF or sham field for 30 min at lower or upper position. Changes in DCF fluorescence intensity are presented as means ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. RWPE treated cells exposed to sham field. PBS, phosphate buffered saline; RWPE, ragweed pollen extract; SMF, static magnetic field.*

These observations suggest that although SMF is not able to decrease ROS production by RWPE directly or lessen the life-span of ROS under cell-free conditions, it still may inhibit ROS entry into living cells or promote elimination of ROS by cellular mechanisms.

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

We have previously demonstrated that intranasal RWPE treatment rapidly increases ROS levels in the lungs of experimental animals prior to the recruitment of inflammatory cells (Boldogh et al. 2005). 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 nearly 3-fold, statistically significant increase in the antioxidant capacity of the BALF samples when compared to PBS challenge (p<0.001) (Figure 20). Immediate exposure to SMF for 30 min after intranasal challenge significantly lowered (M=20.07%, p<0.001) the increase in the total

antioxidant capacity of the airways induced by RWPE treatment (Figure 20). 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.

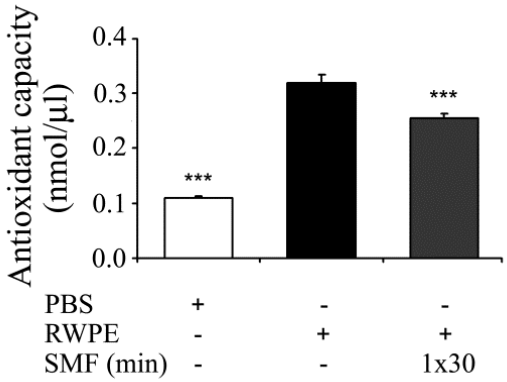

Figure 20. SMF-exposure following intranasal challenge lowers the RWPE-induced increase in total antioxidant capacity of the airways. *Naïve mice were challenged intranasally with RWPE or PBS and immediately thereafter were exposed to SMF or sham field for 30 min. BAL fluid samples were collected 15 min after SMF- or sham field-exposure. Antioxidant potential was measured spectrophotometrically in the supernatant of the samples and expressed in Trolox equivalents. Data are presented as means ± SEM. *** p < 0.001 vs. RWPE challenged, naïve mice exposed to sham field. PBS, phosphate buffered saline; RWPE, ragweed pollen extract; SMF, static magnetic field.*

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 (Chodaczek et al. 2009; Frossi et al. 2003; Suzuki et al. 2005). 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 (M=5.29%, p=0.016, n=50) could only be detected in case of the positive control, histamine (Figure 21). Although all allergens (cat fur, *n*=17; grasses, *n*=13; RWPE, *n*=25), but house dust mite (*n*=14) provoked edema showed a tendency to decrease the diameter upon SMF-exposure (Figure 21). 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.

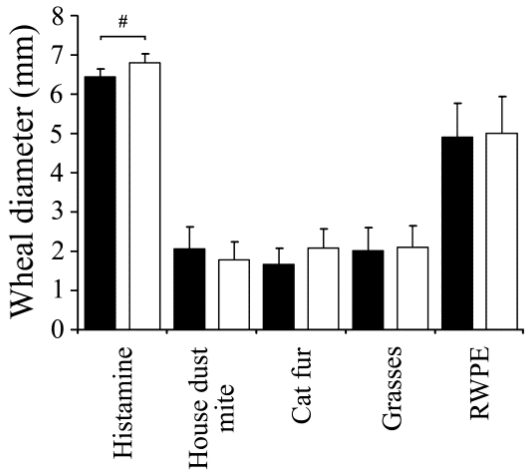

Figure 21. SMF-exposure has no significant direct effect on provoked mast cell degranulation. *Skin prick tests were performed on healthy volunteers as described in Material and Methods. Immediately after introduction of the identical test materials into the skin of both inner forearms, one forearm of the volunteers was exposed to SMF (black bars) while the other one was exposed to sham field (empty bars). The wheal reaction was measured immediately after a 15 min exposure period. Data are presented as means ± SEM.* $^{\#}p < 0.05$.

5. Discussion

Ragweed pollen is one of the most important aeroallergens in many countries since 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, as more than 90% of the ragweed-sensitive subjects have antibodies against this protein (Rafnar et al. 1991). 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 antigens (Tighe et al. 2000). 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 (Allakhverdi et al. 2005; Traidl-Hoffmann et al. 2003). In our work we examined the role of ROS generated by pollen NADPH oxidases both in the sensitization and elicitation phase of allergic inflammation.

Here 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 (Bacsi et al. 2006; Bacsi et al. 2005). Furthermore, our data are also consistent with the more recently observed oxidative stress induction effect of SPPs on monocyte-derived DCs (Pazmandi et al. 2012). Our observation that heat pretreatment, which eliminates pollen NAD(P)H oxidase activity (Bacsi et al. 2006), 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 (Yamada et al. 2006). It has also been demonstrated that complex glucan structures bearing an α(1-3)-fucosylated core, as well as membrane lipid peroxidation products can either directly or indirectly activate the TLR4 signaling cascade (Imai et al. 2008; Tang et al. 2008; Thomas et al. 2003). Because ragweed pollen grains contain α1,3-linked core fucosylated glucans (Wilson et al. 1998) and pollen membranes are susceptible to enzymatic or free-radical-catalyzed peroxidation (McKersie et al. 1990; Mueller 2004), we suppose that these pollen grain components can induce oxidative stress

in DCs via TLR4-mediated mechanism. However, a recent study has reported that TLR4 and its adaptor Toll-IL-1R domain-containing adapter inducing IFN-β (TRIF) signaling and pollen-intrinsic NAD(P)H oxidase activity are not necessary for the induction of allergic airway disease and airway hyperresponsiveness (Shalaby et al. 2013). Nevertheless, future studies are needed to test our hypothesis.

Oxidative stress activates the NFκB and MAPK signaling pathways that are responsible for transcriptional activation of proinflammatory cytokine and chemokine genes in macrophages and DCs, respectively (Riedl and Nel 2008; Verhasselt et al. 1998; Xiao et al. 2003). 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 (Kantengwa et al. 2003). In addition to the increased expression of costimulatory molecules, superoxide anion-treated DCs exhibit enhanced capacity to trigger T cell proliferation (Kantengwa et al. 2003). 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 (Diaz-Sanchez et al. 1999). As TNF-α, IL-6 and CD80/86 are highly involved in the pollen-induced processes it could be taken into consideration that they might act as threapeutical targets mainly in the late phase of the reaction. Anti-TNF-α, anti-IL-6 and CTLA4-Ig have been proved that exert beneficial effects on chronic inflammatory disorders such as rheumatoid arthritis. However, several recent clinical trials have shown either very little or no positive effect during anti-TNF-α therapy in pulmonary diseases. Indeed, in mild to moderate asthmatic individuals, TNF-α antagonism was not effective for preventing allergen-mediated eosinophilic airway inflammation (Mukhopadhyay et al. 2006). A previous study suggested that CTLA-Ig binding to CD80/CD86 molecules transmitted a reverse signal to DCs resulting in the activation of the immunomodulatory enzyme IDO (Grohmann et al. 2002; Munn et al. 2004). These IDO^+ DCs have been proposed to possess strong immune regulatory and tolerance inducing capacity. However, a more recent study suggests that CTLA-Ig exerts

immunoregulating capacity by directly interfering with DC/T cell interaction and is not effective in generating regulatory DCs (Mayer et al. 2013).

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 (Trinchieri 2003). 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 (Allakhverdi et al. 2005). It has recently been reported that oxidative stress can activate the nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) mediated signaling pathway that dominates over the TLR pathways, which promote IL-12 production (Tan et al. 2005). Thus the Nrf2-dependent pathway may be responsible for the suppression of IL-12 generation in DCs (Tan et al. 2005). Furthermore, E_1-phytoprostanes, a class of prostaglandin–like lipid mediators of pollen grains, inhibit the LPS or CD40 ligation induced production of IL-12 (Traidl-Hoffmann et al. 2005). Since E_1-phytoprostanes are formed nonenzymatically via reactive oxygen radicals from α-linolenic acid (Traidl-Hoffmann et al. 2005), 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 (Bacsi et al. 2005). 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 (Xiao et al. 2003). 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 (Xiao et al. 2003). 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, 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 (Allakhverdi et al. 2005). Although, there are differences in the amount of the detected cytokines compared to previous observations (Allakhverdi et al. 2005), the discrepancies may be attributed to the serum-free medium used in our cell cultures or to the different methods applied for re-stimulation.

Our results, showing that after priming with pollen-treated DCs, $CD4^+$ T cells of non-allergic individuals produce higher amount of IFN-γ but release same levels of Th2 cytokines (except IL-3) compared to those from ragweed allergic subjects, are in line with previous observations showing significantly higher percentage of IFN-γ-producing Th cells in normal control subjects than asthmatic patients, while there were no differences in the percentage of IL-4-producing Th cells (Wong et al. 2001). It has also been reported that IFN-γ production of effector T cells generated *in vitro* from naïve precursors from patients with atopic dermatitis is decreased compared to that of healthy T cells (Jung et al. 1999). 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 (Hussein et al. 2009).

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 $CD4^+CD25^+$ regulatory cells; however, its transient expression has also been reported in human activated nonregulatory $CD4^+$ T cells (Wang et al. 2007). Based on this observation, it seems that most of the $Foxp3^+$ cells are comprised by activated T cells developed after *in vitro* stimulation of naïve $CD4^+CD25^-$ T lymphocytes. Since 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 naïve 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 naïve T cells from atopic subjects possess elevated intrinsic ability to differentiate into Tr1 regulatory cells ($CD4^+CD25^{+/-}FOXP3^-IL\text{-}10^+$) after priming with DCs (Akdis et al. 2004). 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 glutathione-S-transferases (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 (Gilliland et al. 2004), and exhibited a more intense nasal response to allergens inhaled with secondhand tobacco smoke as compared to clean air (Gilliland et al. 2006). Future studies performed with DCs from atopic and non-atopic 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 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 (Dharajiya et al. 2007). Because SMF can act on free radicals we have studied whether SMF exposure has an effect on the development of allergic inflammation. Recently external application of static magnetic fields has become popular as complementary therapy in the treatment of inflammatory conditions. Although a number of studies supports a potential therapeutic benefit of pulsed electromagnetic field (PEMF) application to help in the treatment of inflammatory pain conditions (Mert et al. 2014; Prato et al. 2005; Shupak et al. 2004; Shupak et al. 2006), osteoarthritis (Nelson et al. 2013), acceleration of wound healing (Cheing et al. 2014) and the modulation of angiogenesis (Tepper et al. 2004); direct evidence supporting

the therapeutic use of SMF is less established. It is widely published that SMF of certain intensity mainly at mT range promotes bone healing process and bone formation *in vivo* and also increases the osteogenic ability of osteoblasts and inhibit bone resorption capacity of osteoclasts *in vitro* /reviewed in (Zhang et al. 2014)/. Previous investigations of the SMF action on inflammation are based mainly on *in vivo* studies of some diseases such as arthritis (Taniguchi et al. 2004) and management of pain. Formerly, Laszlo et al. have configurated a moderate strenght inhomogeneous SMF generator to achieve the best possible antinociceptive effect in an *in vivo* model of visceral pain. It was found that whole body exposure of mice to inhomogeneous SMF is able to manage to diminish the number and duration of formalin and carragenan-induced nocifensive responses in both acute somatic and inflammatory nociception (Gyires et al. 2008; Laszlo et al. 2007) and reduce inflammatory mechanical hyperalgesia (Antal and Laszlo 2009; Sandor et al. 2007). Fundamental pharmacologic analysis of the same model with the same SMF system was demonstrated that the antinociceptive/analgesic action was over 80% and seemed to be mediated by μ-opioid receptors (Gyires et al. 2008) and inactivation of capsaicin-sensitive sensory fibers (Sandor et al. 2007). More recently the effect of inhomogeneous SMF-exposure on the secretion of different cytokines by human macrophages and lymphocytes was tested *in vitro*. It was demonstrated that SMF has an inhibitory effect on the release of pro-inflammatory cytokines IL-6, TNF-α and IL-8 from macrophages, furthermore SMF-exposure increases the production of IL-10 by lymphocytes. These observations provide a possible background mechanism of the *in vivo* experienced antinociceptive effects in mice (Vergallo et al. 2013).

In this study, we report that exposures to SMF significantly lowered RWPE challenge-induced allergic inflammation in a murine model. Although the precise mechanism by which SMF mediates beneficial effects needs to be elucidated, our novel observation may provide a hint on a future non-invasive therapeutic modality for treatment of human allergic airway inflammation.

We found that the effects of SMF-exposure on allergic inflammation were mediated at least partially by decreasing ROS levels in the airways. Despite that previous observations suggest that pollen-exposure-induced oxidative stress may participate in the initiation of allergic immune responses, the pilot experiment of this study revealed that daily SMF-exposure during the sensitization phase of RWPE-induced allergic reactions did not modify the intensity of the developing airway inflammation. In the experimental allergy model we used, mice were injected intraperitoneally with a mixture of RWPE and

alum to elicit allergic sensitization. Adsorption of the antigen onto the alum-insoluble particles leads to the formation of a depot of the antigen that is released slowly, prolonging the time of interaction between antigen and APCs and allows antigen to be presented in particulate form which appears to be more antigenic and can be efficiently phagocytosed (Kuroda et al. 2013). Although these features are likely determinants of alum adjuvanticity, other mechanisms are also involved. Alum preferentially induces Th2 responses and IgE production via working as DAMP and promoting the release of uric acid and DNA from damaged cells. These agents induce Th2 responses through activation of Syk and PI3 kinase or TBK1-IRF3 in DCs. Furthermore, several studies have reported that alum is able to activate the intracellular stress sensor inflammasomes (Eisenbarth et al. 2008; Li et al. 2007) and ROS are required for inflammasome activation (Zhou et al. 2010). However, the critical role of alum as an adjuvant to activate inflammasomes has been controversial, as alum can exert adjuvanticity also in mice deficient of inflammasomes (Franchi and Nunez 2008; Seubert et al. 2011). A recent study revealed that alum-induced adjuvant effects are dependent on inducible heat shock protein 70 (hsp70) (Wang et al. 2012). Although no data are available on the effects of SMF-exposure on hsp70 expression in murine antigen presenting cells, it has previously been reported that 100 mT SMF-exposure has no significant effect on hsp70 production in NIH3T3 cells (Belton et al. 2009). These observations indicate that the adjuvant effects of alum on hsp70 overcome the ROS-mediated activating signals in antigen presenting cells during the sensitization. It is also worth noting that alum has been used as a safe vaccine adjuvant in humans for many decades. However, there are also some limitations of aluminium-containing adjuvants, which include local reactions, augmentation of IgE antibody responses, ineffectiveness for some antigens and inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses.

Reactive radicals generated by pollen NAD(P)H oxidases induce oxidative stress in the airways within minutes of exposure (Boldogh et al. 2005). Oxidative insult is able to disrupt airway epithelial cell tight junctions (Xu et al. 2013) thus promoting the interaction of allergens with mast cells and also contributing to the recruitment of inflammatory cells in the airways (Boldogh et al. 2005). Challenge with RWPE induces an initial neutrophil recruitment that is followed by eosinophil influx (Schneider et al. 1997) and both cell types are known to contribute to oxidative stress during allergic inflammation (MacPherson et al. 2001). The neutrophil influx starts at 4 h and peaks at 24 h following RWPE challenge (Dharajiya et al. 2007) and as a response to oxidative stress

elevated antioxidant capacity of the airways becomes detectable (Nemmar et al. 2009). 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 (Dharajiya et al. 2007) also inhibited allergic airway inflammation. In a previous study we have demonstrated that scavenging RWPE-generated ROS was able to prevent allergic inflammation in the airways by coadministering antioxidants (Dharajiya et al. 2007). However, scavenging ROS generated by neutrophils recruited at 4 and 24 h failed to do so (Dharajiya et al. 2007). These observations raise the possibility that SMF-exposure either has distinct effects on ROS production and elimination than those of antioxidants (ascorbic acid and N-acetyl cysteine), or it can inhibit the recruitment of inflammatory cells by ROS-independent mechanism(s). Indeed, it has previously been reported that isolated neutrophils, which appear to be highly sensitive to both static and alternating magnetic fields (Poniedzialek et al. 2013 b), under the exposure to strong SMF (0.6-2 T) generated significantly less superoxide anions than detected in controls (Ishisaka et al. 2000). Furthermore, in a recent study a significant decline in ROS production by human peripheral blood neutrophils has been shown after 15 min exposure to SMF (~60 mT), while a longer incubation time (45 min) caused a reverse phenomenon (Poniedzialek et al. 2013 a). The authors conclude that the SMF-exposure may directly modulate the activity of neutrophil NADPH oxidases and they highlight the importance of proper adjustment of exposure time to SMF for any potential therapeutic applications.

SMF-exposure was found not to be 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 (Harkins and Grissom 1994). Experiments have also been carried out with the heme enzymes, horseradish peroxidase, and cytochrome P-450 (as reviewed in (Okano 2008)). 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. Pollen NAD(P)H oxidases generate superoxide anions by transferring electrons from NADPH or NADH to molecular oxygen (Boldogh et al. 2005). Superoxide anions in living cells are converted by superoxide dismutase to hydrogen peroxide molecules, which are eliminated by catalases and glutathione peroxidases (reviewed in (Rahman 2007)). 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 (Kurzeja et al. 2013).

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 (Morris and Skalak 2008). Based on these pharmacological experiments the authors proposed that SMF-exposure may activate L-type Ca^{2+} channels in vascular smooth muscle cells that results in increased intracellular Ca^{2+} levels and induces constriction thus limiting edema formation (Morris and Skalak 2008). Although their work provided a rigorous scientific evaluation of the physiological effects of SMF-exposure on edema in a rat model, it remains to be determined whether SMF-exposure changes vascular tone and/or influences microvascular permeability in human tissues (the possibilities of which were discussed (Ohkubo and Okano 2011). 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 our previous observations that ROS generated by pollen grains-associated NAD(P)H oxidase have no direct impact on IgE-mediated mast cell degranulation (Chodaczek et al. 2009). On the other hand, while

histamine is the major mediator of edema formation, during degranulation, mast cells release other compounds such as tryptase, cathepsin G, TNF and VEGF, which are able to increase vascular permeability /reviewed in (Kunder et al. 2011)/. The effects of these vasoactive mediators could be found behind the phenomenon that the size of the edema usually does not correlate with the concentration of histamine released from activated mast cells, and some patients show no significant histamine release during the immediate phase of allergy as assessed by the microdialysis technique (Horsmanheimo et al. 1996). Based on these observations, varying and/or low levels of released histamine also could be an explanation for the weak impact of SMF-exposure on provoked mast cell degranulation.

Although the same magnetic matrix/matrices were used for SMF generation in all experiments, some potential differences in the SMF-induced effects acting at the target site can be predicted. Namely, the self-motion of an electrically charged object (like mouse) in an external SMF as occurs in the *in vivo* experiments may generate motion-induced currents in its own body. Such a current can influence the distribution of specific cells in the lung, but hardly any induced current can occur in cells of the *in vitro* experiments. In the human trial induced currents can be generated without self-motion of the forearm in the exposure chamber of the SMF generator. The motion of electrically charged cells within the blood vessels of the forearm manifests a source of induction. This motion-induced effect may influence the observed biological responses to SMF-exposure. We have a dual solution to this argument. First, no results were compared between different types of measurements (*in vitro*, *in vivo*, and human). Second, the effect of induced currents under specific SMF-exposure conditions provided here for the *in vivo* experiments has been estimated not to affect the physiology of mice significantly as previously discussed (Laszlo and Gyires 2009). Even if it did, this fact would not counteract the merit of SMF-exposure in the clinical application, since homogeneous magnetic induction up to 8 T is accepted not to influence blood circulation (Kangarlu et al. 1999) and this complies with the present guidelines of magnetic resonance imaging (MRI) safety (Protection 2009).

MRI was agreed not to be regarded as a purely diagnostic device (Laszlo et al. 2009; Schenck 2005), its SMF component (B_0) exerts finite forces in the living body and accordingly, invokes such responses that may be observable, perceptible through changes in the (patho)physiology. While the direction of the dominant component of the SMF (B_0) within the tunnel of a conventional MRI is *superior-inferior*, mice in our *in vivo*

experiments were exposed to a *ventral-dorsal* SMF in the inhomogeneous SMF generator. Fringe SMF around functioning MRI devices are typically gradient SMF and their magnetic induction might exceed that of the geomagnetic field by orders of magnitude. Furthermore, magnetic induction components of this fringe SMF are greater than those of Earth in all directions. Focusing on the internal side of an MRI, our experimental arrangement resembles more to an open field MRI. However, while humans in and around MRI devices are exposed to SMF with their whole body, only local exposure to a strong inhomogeneous SMF was used in the present human trial. 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, 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.

Összefoglalás

A parlagfű pollen az allergiás megbetegedések – mint a rhinitis, kötőhártya-gyulladás és az allergiás asthma – egyik leggyakoribb és a legsúlyosabb tüneteket előidéző tényezője Közép Európában. Az allergiás megbetegedések patomechanizmusának számos részlete már jól ismert, de a folyamatokat elindító lépésekről még keveset tudunk.

A dendritikus sejtek (DS-ek) kulcsfontosságú szereppel bírnak az allergiás reakciók szenzitizációs fázisában, így munkánk során először a pollenszemek által termelt reaktív oxigéngyökök monocita-eredetű DS-re gyakorolt hatását vizsgáltuk. Eredményeink azt mutatják, hogy a pollen-expozíció oxidatív stresszt váltott ki a DS-ekben, melynek kialakulásához a NAD(P)H oxidázok mellett a pollenszemek egyéb komponensei is hozzájárultak. A megemelkedett intracelluláris reaktív oxigéngyök szint elősegítette az IL-8, a TNF-α valamint az IL-6 citokinek termelődését. A pollenszemekkel történt kölcsönhatást követően a DS-ek felszínén fokozódott a CD80, CD86, CD83 és HLA-DR molekulák kifejeződése. A pollenkezelt DS-ek elősegítették a naïve T sejtek különböző citokineket (IL-3, IL-5, IL-10, IFN-γ) termelő effektor T sejtekké történő differenciálódását. Az antioxidáns hozzáadása a tenyészethez gátolta a DS-ek fenotípusos és funkcionális változásait.

Ismert, hogy az oxidatív stressz hozzájárul az allergiás gyulladás kialalkulásához. Bizonyított, hogy a statikus mágneses mező (SMM) által kifejtett biológiai hatások részben szabadgyökök által mediáltak, ezért a továbbiakban közepes erősségű SMM hatását vizsgáltuk az allergiás légúti gyulladás egérmodelljében, valamint humán allergiás bőrteszt vizsgálatokban. Kimutattuk, hogy már egyetlen, 30 perces SMM expozíció közvetlenül az intranazális parlagfű pollenkivonat kezelés után jelentősen mérsékelte a légutak teljes antioxidáns kapacitásának pollen-indukálta növekedését, valamint szignifikánsan csökkentette az allergiás gyulladás mértékét is. Ismételt (3 egymást követő napon) vagy megnövelt időtartamú (60 perces) SMM kezelés a nazális pollenkivonat adását követően hatékonyabban csökkentette az allergiás reakciókat, mint az egyszeri 30 perces kezelés. A SMM expozíció sejtmentes körülmények között nem befolyásolta a pollenkivonat szabadgyök-termelő képességét, míg a pollenkivonat által indukált reaktív oxigéngyökök szintjét légúti hámsejtekben csökkentette. A humán allergiás bőrtesztek

eredményei szerint a SMM expozíciónak nincs közvetlen szignifikáns hatása a hízósejt degranulációra.

Eredményeink a pollen eredetű oxidatív stressz allergiás gyulladás szenzitizációs és kiváltási fázisában betöltött fontos szerepére hívják fel a figyelmet, valamint hozzájárulhatnak új terápiás eljárások kidolgozásához.

7. References

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7.2 Publication list prepared by the Kenézy Life Sciences Library

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Candidate: Anikó Csillag

Neptun ID: XTMLWO

Doctoral School: Doctoral School of Molecular Cell and Immune Biology

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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.
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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.
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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.
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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.
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8. Keywords

Dendritic cell, ragweed pollen, oxidative stress, static magnetic field, allergic airway inflammation

Tárgyszavak

Dendritikus sejt, parlagfű pollen, oxidatív stressz, statikus mágneses mező, légúti allergiás gyulladás

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