Fakultät für Medizin

Zentrum für Allergie und Umwelt (ZAUM)
Institut für Allergieforschung (IAF)

Analysis of the sensitizing properties of ragweed pollen components and the influence of environmental factors in a murine *in vivo* model

Maria Kamml

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften
(Dr. rer. nat)

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. Carsten Schmidt-Weber

Prüfer der Dissertation:

1. Prof. Dr. Jan Gutermuth
2. Univ.-Prof. Dr. Jörg Durner

Die Dissertation wurde am 21. 11. 2013 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 07. 05. 2014 angenommen.

Content

Figures

Figures

Tables

Abbreviations

ADA	Adenosine deaminase
ADO	Adenosine
AHR	airway hyperresponsiveness
AID	activation induced-cytidine deaminase
$Al(OH)_3$	aluminum hydroxide
alum	aluminum hydroxide
APC	antigen presenting cell
APRIL	a proliferation-inducing ligand
BAFF	B cell activating factor
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BSA	bovine serum albumin
CD	cluster of differentiation
CLN	cervical lymph node
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
HE	Hematoxylin and eosin stain

Abbreviations

H_2O	water
H_2SO_4	sulfuric acid
HCl	hydrochloric acid
HRP	horseradish peroxidase
i.n.	intranasal
i.p.	intraperitoneal
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KCl	potassium chloride
kDa	kilo Dalton
KO	knockout
KOH	potassium hydroxide
LN	lymph node
LPS	lipopolysaccharids
LTB_4	leukotriene B4
MHC	major histocompatibility complex
MLN	mesenteric lymph node
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NHS	*N*-Hydroxysulfosuccinimide
NK	natural killer
NKT	natural killer T cell
OVA	ovalbumin

Abbreviations

O_3	ozone
PALM	pollen-associated lipid mediator
PAS	Periodic Acid Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGE_2	prostaglandin E2
q. s.	quantum satis/sufficit
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RWE	ragweed pollen extract
SD	standard deviation
SEM	standard error of the mean
Th1	T helper type 1 cell
Th2	T helper type 2 cell
TNF	tumor necrosis factor
Treg	regulatory T cells
Tris	tris(hydroxymethyl)aminomethane
TSLP	thymic stromal lymphopoietin
ULPC	Ultra Performance Liquid Chromatography

Abstract

Common ragweed (*Ambrosia artemisiifolia*) is a widely spread allergenic weed of the Asteraceae plant family and was introduced in Europe from Northern America. Its pollen is a strong elicitor of allergic airway inflammation and one of the main causes of allergic reactions in late summer and autumn. Upon contact with the respiratory mucosa, pollen release not only allergens, but also non-protein substances (e.g. adenosine) that exert proinflammatory as well as immunomodulatory effects. However, the precise contribution of single substances and the mechanisms that underlie the strong allergenicity of ragweed pollen is still not known. Additionally, meteorological changes caused by climate change and alterations in the concentration of greenhouse gases, like carbon dioxide and ozone, can influence the production and allergenic content of pollen as well as the growth and distribution of pollen producing plants. This may result in changes in the prevalence and severity of allergic diseases.

The objective of this study was to establish a physiological murine *in vivo* system to evaluate specific sensitizing and non-specific proinflammatory properties of ragweed pollen as an environmentally relevant allergen.

Therefore, two different models of induction of ragweed-specific allergic airway inflammation were developed. The first model induces sensitization by intraperitoneal injection of ragweed extract in combination with aluminum hydroxide followed by three intranasal challenges, leading to a strong and robust ragweed-specific Th2 sensitization and elicitation of ragweed-specific allergic airway inflammation. The second model mimics the physiological way of exposure to ragweed pollen via the nasal mucosa without any additional adjuvant, by eleven consecutive intranasal instillations with ragweed pollen extract. This novel model of sensitization leads to strong cell infiltration into the lung, together with mucus production, and a Th2-cytokine signature in pulmonary tissue.

Using the intraperitoneal sensitization model the allergy-inducing properties of ragweed pollen grown under elevated ozone conditions were evaluated. However, there were observed no differences between mice treated with pollen grown under elevated ozone conditions compared to mice treated with control pollen, neither in the sensitization nor in the elicitation phase of allergic airway inflammation. The more physiological intranasal sensitization model showed that allergic airway inflammation was mediated by the protein containing total ragweed extract, but not by the major allergen Amb a 1 alone or the protein-free fraction of the ragweed extract. However, also the presence of small molecular non-

protein compounds like adenosine is crucial for a strong sensitization. Further, we could show that intranasal ragweed pollen extract augments a preexisting bystander inflammation. In conclusion, these findings revealed that protein as well as non-protein substances play a critical role in sensitization.

Zusammenfassung

Das beifußblättrige Traubenkraut (*Ambrosia artemisiifolia*) ist eine weitverbreitete allergene Pflanze aus der Familie der Korbblütler und wurde von Nordamerika in Europa eingeschleppt. Sein Pollen ist ein starker Auslöser von allergischen Atemwegsentzündungen und eine der Hauptursachen für allergische Reaktionen im Spätsommer und Herbst. Beim Kontakt mit der Schleimhaut der Atemwege werden von den Pollen nicht nur Allergene, sondern auch nicht-proteinhaltige Substanzen (z.B. Adenosin) freigesetzt, die sowohl proinflammatorische als auch immunmodulatorische Effekte ausüben. Jedoch sind die genaue Bedeutung der Einzelsubstanzen und die Mechanismen, die für die starke Allergenität des Ambrosiapollens verantwortlich sind, noch nicht bekannt. Zusätzlich können durch den Klimawandel bedingte meteorologische Veränderungen in der Konzentration von Kohlenstoffdioxid und anderen Treibhausgasen, wie Ozon, sowohl Wachstum und Verbreitung der Pflanzen als auch die Produktion und den Allergengehalt der Pollen beeinflussen. Dies kann zur Veränderung in der Prävalenz und der Schwere von allergischen Erkrankungen führen.

Ziel dieser Studie war ein physiologisches murines *in-vivo*-Model zu etablieren, um spezifische sensibilisierende und nicht-spezifische proinflammatorische Eigenschaften des Ambrosiapollens als umweltrelevantes Allergen zu bewerten.

Hierfür wurden zwei verschiedene Modelle zur Induktion einer allergischen Atemwegsentzündung entwickelt. Im ersten Modell wird die Sensibilisierung durch die intraperitoneale Injektion von Ambrosiapollenextrakt zusammen mit Alumiumhydroxid mit anschließender intranasaler Provokation ausgelöst. Dies führt zu einer starken Ambrosia-spezifischen Th2 Sensibilisierung und zur Auslösung einer Ambrosia-spezifischen Atemwegsentzündung. Das zweite Modell ahmt die physiologische Art der Pollenexposition über die Nasenschleimhaut ohne zusätzliches Adjuvans nach, indem der Ambrosiapollenextrakt an elf aufeinander folgenden Tagen instilliert wird. Dieses neuartige Modell der Sensibilisierung führt zu einer starken Zellinfiltration mit Schleimproduktion und zu einer Th2-Zytokin-Signatur im Lungengewebe.

Mit Hilfe des intraperitonealen Sensibilisierungsmodells wurden die Allergie-auslösenden Eigenschaften von Ambrosiapollen, die unter erhöhten Ozonbedingungen gewachsen waren, bewertet. Jedoch zeigten sich weder in der Sensibilisierungs- noch in der Auslösephase der allergischen Atemwegsentzündung Unterschiede zwischen den Mäusen, die mit Pollen behandelt wurden, die unter erhöhter Ozonkonzentration gewachsen waren, im Vergleich zu Mäusen, die mit Kontrollpollen behandelt wurden. Mit Hilfe dieses physiologischeren

intranasalen Sensibilisierungsmodells konnten wir zeigen, dass die allergische Atemwegsentzündung vor allem durch den proteinhalten Gesamtpollenextrakt, nicht aber durch das Hauptallergen Amb a 1 alleine oder die protein-freie Fraktion des Pollenextraktes vermittelt wird. Aber auch die Anwesenheit von kleinen, nicht-proteinhaltigen Substanzen wie Adenosin ist für eine starke Sensibilisierung von entscheidender Bedeutung. Weiter konnte gezeigt werden, dass die intranasale Gabe von Ambrosiapollenextrakt zu einer Verstärkung einer vorherbestehenden Entzündung führt.

Zusammenfassend zeigen diese Ergebnisse, dass sowohl Proteine als auch Nicht-Proteine bei der Sensibilisierung eine kritische Rolle spielen.

1 Introduction

1.1 Allergy

In 1906 Clemens von Piquet introduced the term “allergy”. He recognized that hypersensitivity reactions as well as protective immunity were induced from an external agent. Therefore, he suggested for this phenomenon the term “allergy”, from the Greek word allos (‘other’) and ergon (‘work’), describing a specific altered responsiveness. Although Piquet thought of an uncommitted biologic reaction including hypersensitivity as well as attenuated immunological reactions, with the passage of time the term became corrupted and today is used to describe only hypersensitivity reactions [1-3]. In 1963, Coombs and Gell differentiated four distinct types of hypersensitivity reactions (Type I-IV), which lead to damage of tissue. Whereas type I to III are mediated by antibodies, type IV reactions are elicited by activation of T cells. The following study is limited to type I hypersensitivity reactions, which include allergic rhinitis, allergic asthma, and systemic anaphylaxis reactions and are mediated through IgE and mast cell activation. Immediate hypersensitivity reactions involve a sensitization phase (which shows no clinical symptoms), and after a reexposure to the allergen an early-phase reaction (takes place within minutes after allergen exposure) followed by a late-phase response, which develops 2-6 hours after allergen exposure and peaks after 6-9 hours [4]. The early phase reactions are characterized by release of mast cell mediators, which results in wheezing, sneezing, rhinorrhoea and conjunctivitis, whereas the symptoms of the late phase response are caused by an influx of eosinophils and T helper 2 (Th2) cells [5]. Allergic diseases have increased over the past decades, especially in industrialized countries. This increase is discussed controversially and is considered to be due to various reasons, for example the decline of infectious diseases, genetic predispositions, lifestyle changes or air pollutions [6, 7].

1.1.1 The mechanism of allergic sensitization and inflammation

In brief, in atopic subjects antigen exposure leads to an antigen-specific activation of naïve CD4 T cells through dendritic cells. Subsequently, the naïve T cells differentiate into T helper 2 (Th2) cells, which drive B cell differentiation into IgE producing plasma cells. Secreted IgE antibodies bind to the Fc receptors (FcεRI) of mast cells. These processes are known as allergic sensitization and do not cause clinical symptoms. After reexposure to the same antigen, IgE antibodies bound to FcεRI on mast cells are cross linked by the allergen, which

leads to degranulation of mast cell vesicles and causes the subsequent pathologic reactions of the allergic response.

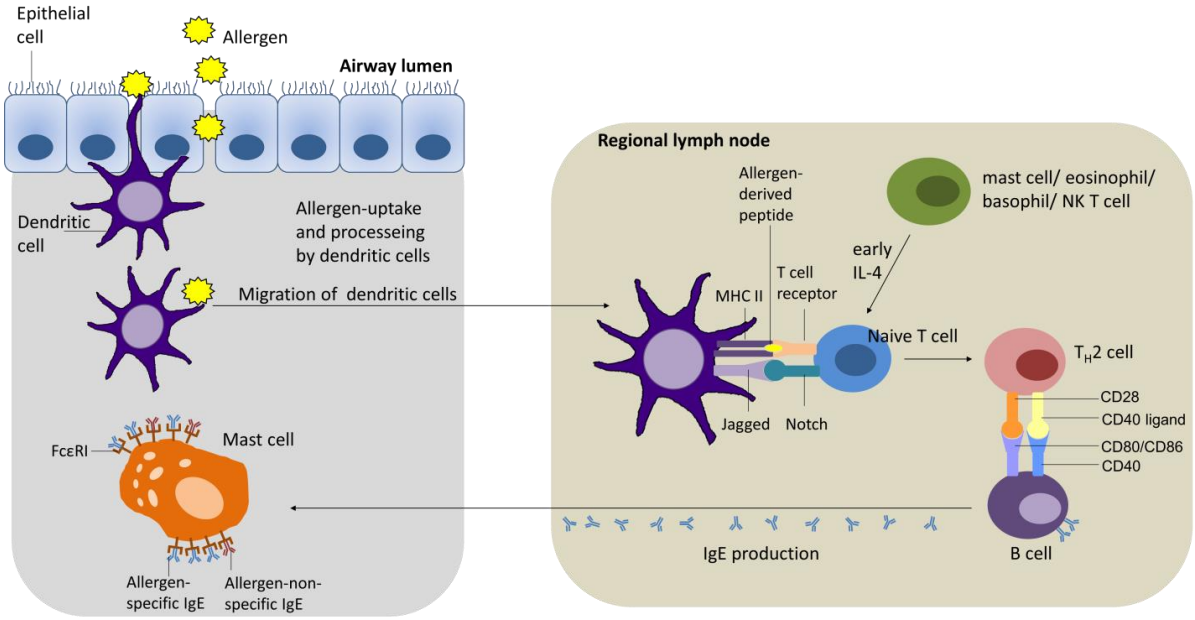

Figure 1: Sensitization to allergens in the airways (adapted from Galli et al., 2008 [8])

1.1.2 Activation of T cells in the allergic response

CD4 T cells (also known as T helper cells) play a major role in the development of adaptive immune reactions. Naïve CD 4 cells may differentiate into distinct subsets of CD4 effector cells (Th1, Th2, T_{reg}, Th17, Th9, and Th22) in response to different antigens, costimulators and cytokines [9-13]. Allergic inflammation is predominantly regulated by Th2 cells, which are also involved in immunity to parasites [14]. For development of a Th2 response towards an allergen in the airways, the allergen is either sampled by dendritic cells in the airway lumen [15] or enters the tissue though disrupted epithelium [16]. Usually allergens are proteins or are bound to proteins. Some have proteolytic activity, like the house dust mite allergen Der p 1, which is a cysteine protease [17]. These proteases are able do directly disrupt epithelial tight junctions and therefore reduce epithelial barrier functions [18, 19]. After entering the airway tissue, allergens are taken up and processed by dendritic cells, which then migrate to local lymph nodes. There, they present peptides of the allergen in context of the major histocompatibility complex (MHC) class II molecules to naïve T CD 4

cells, which bind with their T cell receptor to the MHC II–peptide complex [20]. The differentiation of antigen-stimulated T cells into Th2 cells is depended on "early IL-4", derived from basophils, mast cells, eosinophils, natural killer cells and T cells [21, 22]. IL-4 up regulates the expression of the transcription factor STAT6 and GATA3 [23, 24]. Additionally, T cell receptor signaling induces GATA3 expression [25]. This pathway leads to induction of Th2 cell responses and to further production of IL-4, which amplifies this response and inhibits the development of Th1 and Th17 cells [26]. This process is enhanced by the engagement of Notch on the surface of T cells with Jagged on dendritic cells (Figure 1) [27]. Th2 cells produce various cytokines with different effector functions, mainly IL-4, IL-5, IL-9 and IL-13 [28-30].

1.1.3 **Activation of B cells and class switch recombination**

Stimulated by mainly IL-4 and binding of CD28 [31] and CD40 ligand [32] on T cells to CD80/CD86 or CD40 respectively on B cells, immunoglobulin class switch recombination (CSR) in B cells occurs, leading to the production of IgE.

IgE consist, like all other antibody classes, of two identical heavy chains and two identical light chains and exists in a membrane-bound and a secreted form. Both, heavy and light chains possess an amino terminal variable (V) region that is responsible for antigen recognition and a carboxyl-terminal constant (C) region. The C region of the heavy chain defines the isotype of the antibody. The heavy chain of IgE includes four constant domains (Cε1, Cε2, Cε3, Cε4), and one V domain, the light chain is composed of one V region Ig domain and one C region Ig domain. The V region of the heavy chain (V_H) and the adjoining region of the light chain (V_L) form the antigen-binding site. Heavy and light chains are covalently linked by disulfide bonds (Figure 2) [33].

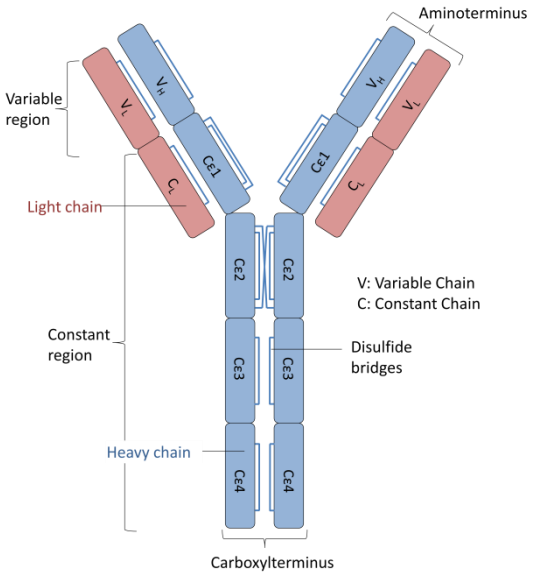

Figure 2: The structure of IgE (adapted from Gould et al.; 2009 [33])

Antibodies are important mediators in humoral immunity. The heavy chains of IgM, IgD, IgG, IgA and IgE antibody isotype classes are encoded by distinct exon clusters, which are organized in the order Cμ, Cδ, Cγ, Cε and Cα. For production of IgE a functional IgE gene has to be assembled. This process takes place in two steps. First, different variable (V) region gene segments are rearranged with diversity (D) and joining (J) gene segments, with addition or removal of random nucleotides at sites of recombination. This so called V(D)J cassette encodes for the antigen specific domain and is situated upstream of the (C) μ exon and allows therefore the production of the μ-heavy-chain protein for IgM. V(D)J recombination talks place during the pre-B cell stage [34, 35]. The second step is called class switch recombination (CSR) and leads in appropriately stimulated B cells to a change of the isotype of the produced antibody (for example from IgM to IgG or IgE), while keeping their antigen specificity (Figure 2). Without stimulus from T cells (like IL-4 and CD40 ligand) B cells produce IgM. After encountering T cell help, IgM expressing B cells undergo isotype class switching, leading to the production of different heavy chain classes, such as γ, ε, or α and therefore the production of IgG, IgE, or IgA. For the production of IgE, the molecular mechanism of isotype switching leads to the excision of a large piece of genomic DNA, spanning from the μ switch sequence (Sμ) to the Sε sequence [36]. The enzyme activation-induced cytidine deaminase (AID) catabolizes the deletion-recombination reaction where the switch regions Sμ and Sε pair to their homologous sequence [37]. After ligation of the VDJ sequence to the Cε locus a complete ε heavy chain is formed and B cells can produce IgE antibodies (Figure 3).

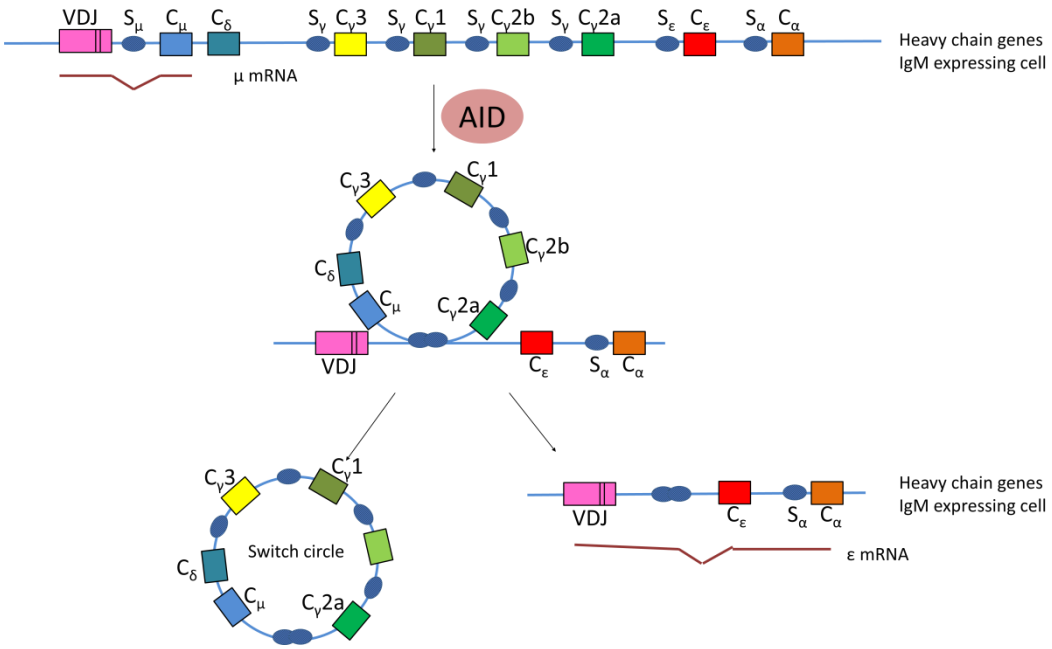

Figure 3: IgE class switch recombination (adapted from Xu et al., 2012 [38])

1.1.4 Activation of mast cells mediator release

IgE antibodies enter the lymphatic vessels and blood circulation, are distributed systemically and bind to the high-affinity receptors for IgE (FcεRI) of tissue-resident mast cells. Besides mast cells, circulating basophils and eosinophils express the FcεRI and are able to bind IgE, and therefore mast cells, basophils and eosinophils are the effector cells of immediate hypersensitivity reactions [39, 40].

Mast cell activation occurs by a reexporsure to an antigen, which leads to cross linking of specific IgE antibodies bound to FcεRI on mast cells. Activation of mast cells leads to three different classes of biological response: secretion of preformed contents of cytoplasmatic granules by exocytosis, synthesis and secretion of lipid-derived mediators, and synthesis and secretion of cytokines, chemokines and growth factors (Figure 4) [41].

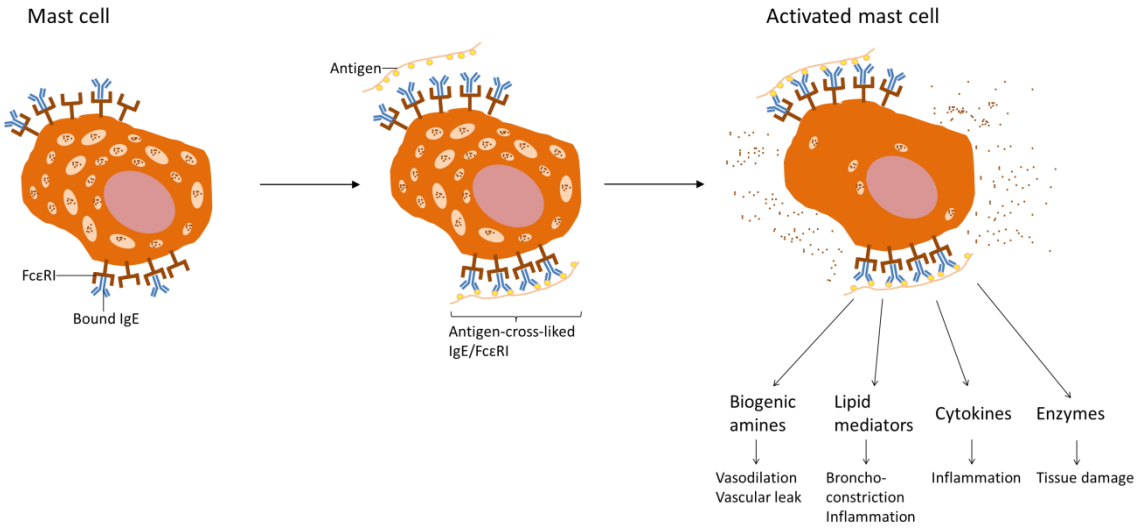

Figure 4: Degranulation of mast cells (adapted from Abbas [42])

For the secretion of preformed mediators the cytoplasmatic granules fuse with the membrane of mast cells and release their content into the external environment. One class of substances stored in and released from cytoplasmatic granules are biogenic amines, which are low molecular weight compounds containing an amine group. In human mast cells histamine is the most frequent and important biogenic amine, but in rodent mast cells also serotonin seems to play an important role [43]. Histamine exerts short lived actions via histamine receptors, because it is removed quickly from the extracellular space by amine-specific transporters [44]. Furthermore, the cytoplasmic granules of mast cells contain neutral serine proteases (such as tryptase and chymase), which lead after releasing to tissue damage [45]. Proteoglycans, including heparin and chondroitin, are strong negatively charged and are important for storing positively charged biogenic amines, proteases and other mediators in the granules. After degranulation proteoglycans may control the kinetic of hypersensitivity reactions, because biogenic amines are released much faster from the proteoglycans than proteases [46].

Besides exocytosis of granules, mast cell activation by the aggregation of FcεRI leads to *de novo* synthesis of a variety of substances, like lipid-derived mediators. Most of them are metabolized from arachidonic acid by cyclooxygenases and lipoxygenase activity. The most frequent mediators synthetized by these pathways are prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4) [47]. Mast cells release also many different inflammatory cytokines, like TNF, IL-5, IL-4, IL-6, IL-13, CCL3, and CCL4 [48-50].

All this performed or newly synthesized mediators secreted by mast cells lead to acute symptoms of immediate hypersensitivity, which can be locally or systemically. Typical local

reactions are bronchoconstriction of the airways, vasodilation, increased vascular permeability, and increased mucus production. The rapid systemic release of mediators can lead also to anaphylaxis.

1.1.5 Chronic inflammation and allergic asthma

Repetitive or persistent allergen exposure can lead to chronic inflammation in sensitized individuals. Chronic inflammation is characterized by infiltration of innate immune cells like neutrophils, monocytes, and macrophages, as well as adaptive immune cells (T and B cells) into the affected tissue, and can lead to allergic asthma. There are allergic (intrinsic) and non-allergic (extrinsic) forms of asthma, but mostly asthma is associated with Th2-type responses and is dependent on T-cell-driven cell recruitment and mediator release by mast cells, eosinophils, and basophils [51]. Allergic asthma is induced by repeated early and late phase reactions, which lead to the repeated and reversible attacks of asthma with chronic inflammation of the respiratory tract. In bronchial biopsies from asthmatic patients infiltrated eosinophils, activated T cells and mast cells were found. Characteristic structural changes in asthma are thickening of the airway smooth-muscle cell layer and collagen deposition under the epithelium (known as basement-membrane thickening) which result both from cell hyperplasia and hypertrophy [52]. The chronic inflammation is mediated by enhanced expression of inflammatory proteins, like adhesions molecules, chemokines, and cytokines. Apart from typical Th2 cytokines like IL-4, IL-5, and IL-13, Th17 cell-derived cytokines such as IL-17A and IL-17F are expressed in the airways of asthmatics and are correlated with the severity of asthma [53]. Another cytokine secreted by Th17 cells, IL-22, which is structurally similar to IL-17, is also present in sera of asthma patients. Interestingly, it was shown that levels of IL-22 in BALF of asthmatic subjects were inversely correlated with levels of proinflammatory chemokines, suggesting a protective role of IL-22 in asthma [54]. Besides that, asthma is a complex disease depending on interaction of genetic and environmental factors. It was shown that the probability to develop allergies or asthma is influenced by the inheritance of several genes. Most of these susceptibility genes are associated with innate immunity and immunoregulation, Th2 cell differentiation, epithelial and mucosal immunity, or lung function and airway remodeling [55-58] . Besides these predisposing genetic factors, environmental factors play an important role in development of allergic asthma. Environmental factors, like air pollutants, tobacco smoke or endotoxins are able to trigger the development of asthma in genetically susceptible individuals [59, 60]. The typical clinical symptoms of allergic asthma are breathlessness, wheezing and coughing,

chronic bronchial inflammation, bronchial smooth muscle hypertrophy and hyperactivity to bronchoconstrictors. Additionally, mast cells develop in increased numbers in the smooth muscle, present there IgE bound to FcεRI and release their mediators after crosslinking by allergens. This local distribution of mast cells plays a major role in development of airflow obstruction and airway hyper responsiveness [61, 62]. In asthmatic patients there is a complex interaction between recruited and tissue-resident immune cells, the structural cells of the lung (e.g. fibroblasts, myofibroblasts and airway smooth muscle cells) and the epithelial cells. Therefore, inhaled antigen leads to enhanced signaling between the airway epithelium and underlying structural and immune cells, producing a microenvironment, which leads to facilitated allergic sensitization, sustained Th2 cell-associated inflammation, and tissue remodeling. Taken together this leads to reduced lung function [63]. It is also important to mention that infections with common respiratory viruses such as rhinoviruses and influenza viruses can be predisposing factors for the development of asthma as well as lead to exacerbations of existing asthma [64, 65].

1.2 Pollen

Airborne pollen is the reason for many respiratory allergies. In general, pollen consists of powdery grains and is produced by the anthers of seed bearing plants. Pollen of different species shows a wide variety of different shapes and is sized from 10 μm to 100 μm. Pollen grains have a strong wall, which consists of two layers, the inner intine wall (which consists mainly of cellulose) and the outer exine wall (which consists mainly of sporopollenin). As each pollen grain results from meiosis it is haploid. The pollen grains harbor the male gametophyte and transport them to the female pistil of the plant. After landing on the stigma, the pollen hydrates, germinates, and extends a tube, that enters and fertilizes the ovule [66].

1.2.1 **Allergens in pollen**

In general, some important aspects of allergens on their allergenicity are solubility, stability, size, and the compactness of the protein folding. In most cases the typical characteristics of allergens are relatively small molecular weight, hydrophilic properties, and negative charge. Furthermore, posttranslational modifications, like glycosylation or hydroxylation, can have an important impact on allergenicity be influencing the stability of a protein. However, even if structural features of allergens seem to be essential in determining the direction of an immune response, still it is not clear why some individuals develop specific IgE against a

given protein, while others do not [67]. The importance of allergens but also of non-allergenic compounds is reviewed by Gilles et al. [68].

Allergens are the most important agents in allergic sensitization towards pollen. In this context it was shown, that pollen count and allergen in ambient air follow the same temporal trends and that the approximated daily exposure with Bet v 1, the major allergen of birch, is in the low nanogram range [69, 70].

1.2.2 Adjuvant factors released by pollen

Pollen is a complex biological unit of many different proteins and lipid derived substances. Therefore, it is important to consider that pollen do not only function as carriers of allergens, but also release a multitude of other different substances upon contact with the moist environment of the airways. Pollen allergens are sampled and processed by antigen presenting cells and then presented to T cells leading to activation of a Th2-based adaptive immune response, as described before. However, under natural conditions, individuals are never exposed to purified allergens, but rather to whole pollen grains or to a complex mixture of pollen derived substances, which can have adjuvant as well as proinflammatory or immunomodulatory effects.

One of the most important adjuvant factors derived from pollen is the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase. The general function of NADPH oxidase is to generate reactive oxygen species. NADPH oxidases contained in plants do not differ much from those in mammalian cells. Their biological function is regulation of different vital functions like growth and development as well as defense from pathogens [71, 72]. It was shown that ragweed pollen possesses intrinsic NADPH oxidase activity, which induces oxidative stress as well in cultured epithelial cells as in lung tissue *in vivo* and augmented allergic airway inflammations by promoting neutrophil recruitment [73].

Another class of substances released by pollen are bioactive lipids. The physiological role of these lipids is to help the pollen tube to penetrate the stigma and direct the growth of the pollen tube. Therefore, they play a crucial role in the plant fertilization process [74]. Upon contact with an aqueous phase, pollen release significant amounts of eicosanoid-like substances, which were determined by means of cross reactivity in ELISA as leukotriene B_4-like and prostaglandin E_2-like substances, and therefore show structural and functional homology with eicosanoids. [75]. As eicosanoids are well known to affect innate as well as adaptive immune responses in humans, the effects of these eicosanoid-like substances (or so-called pollen-associated lipid mediators (PALMs)) were evaluated in many studies. PALMS

were shown to activate and recruit polymorph nuclear granulocytes (PMNs) *in vitro* [76] and to mediate migration and activation of eosinophils [77]. Furthermore, PALMs affect the migration and inhibit the release of IL-12 by human dendritic cells, which increase their capacity to augment T helper 2 cell polarization [78]. Additionally, it was shown in a murine *in vivo* model that water soluble substances released from pollen lead to a preferential induction of Th2 responses [79]. Recently it was shown that pollen from birch and other species release high amounts of nucleosides, especially of adenosine. Adenosine conducted tolerogenic signals, by modulation of dendritic cell function and inhibition of Th1 responses [80].

1.3 Ragweed

1.3.1 **Ecology of ragweed plants**

Ragweed (*Ambrosia artemisiifolia*) is an annual weed, belonging to the Asteraceae family. Originally, it is native in Northern America, but now it spreads not only in southern and southeastern parts of Europe, but also in areas with cooler climatic conditions like Germany. It is an erect growing plant, with usually strongly ramified and haired upper parts. Normally, the leaves show a bipinnate pattern. With male and female flowers on the same plant it is monoecious and spreads by distribution of seeds approximately ten weeks after its flowering season. The numerous male capitula are located in leafless racemes, the female capitula are arranged either alone or in small groups in the shoulder of the upper leaves. Ragweed plants show numerous side-trims, the ramification starts at about 2-4 cm above floor level at the first to the third node. Usually it is sized from 20 to 120 centimeters, but the height is strongly depended on the environment, especially on water supply and competition (Figure 5 A-C). Ragweed grows best in warm and moist conditions, but does not grow well in dry periods or at low temperatures. In Germany ragweed starts flowering in June and can last till November, the main flowering season is between August and October [81].

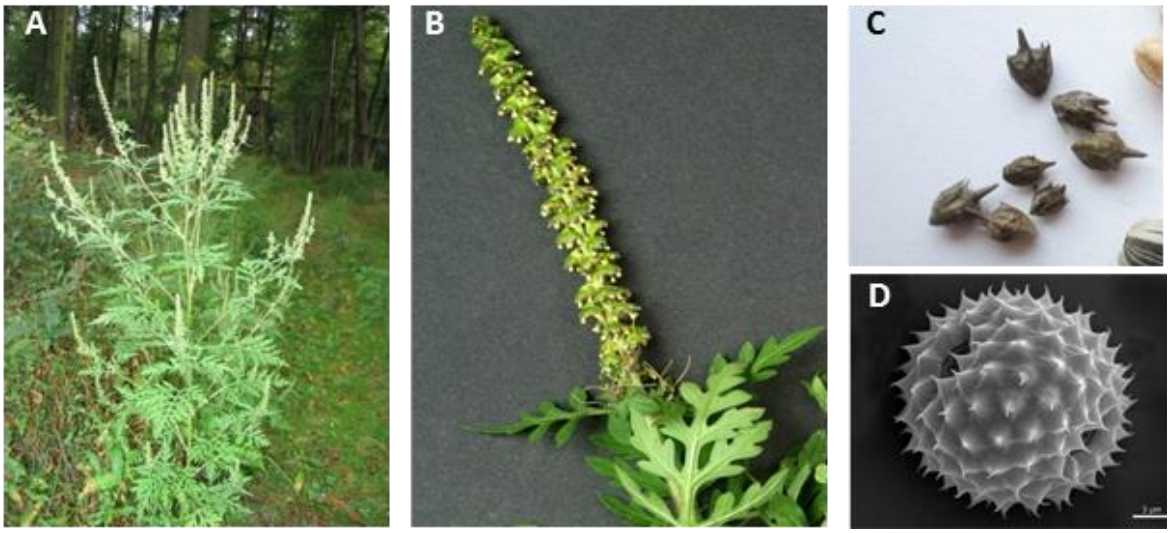

Figure 5: Ragweed (A-C courtesy from Heidrun Behrendt; D from Ingrid Weichenmeier)
A) Ragweed plant; B) Ragweed flowers; C) Ragweed seeds; D)Electron microscopy of ragweed pollen

1.3.2 **Ragweed pollen**

With a size of 18-22 µm ragweed pollen grains are small (Figure 5D) and are frequently involved in episodes long-distance transport [82]. Ragweed pollen is known to evoke strong allergic reactions. Different studies name various threshold levels for the dose of ragweed pollen, which are able to induce symptoms in allergic patients. Levels reported differ between 1-3 grains/m^3 to 10-50 grains/m^3 and seem to be dependent on the individual sensitivity of every patient. However, very low concentrations of pollen grains are able to trigger allergic reactions ins sensitive patients and may lead to severe allergic asthma [83].

The highly allergenic ragweed pollen contains a complex mixture of many different proteins, which include major, minor and panallergens. Major allergens are characterized by the majority of reactive patients displaying IgE antibodies towards the allergen (more than 50% by definition), whereas minor allergens are recognized by only a limited number of sensitive individuals. Often minor allergens are involved in general vital functions and therefore are found in a multitude of different plants and share highly conserved sequence regions and structures. They have been shown to be responsible for cross-reactions of unrelated plant species. As they are distributed in unrelated organisms these allergens often are called “panallergens”. Known panallergens are originated in few protein families, including profilins, polcalcins, and non-specific lipid transfer proteins [84].

So far, 10 different ragweed pollen allergens have been characterized (Table 1). The most important major allergen in ragweed is Amb a 1, formerly known as antigen E. In skin tests

95% of ragweed-sensitive individuals show a reaction on Amb a 1 and about 13% of their total serum IgE is specific for Amb a 1. It is a nonglycosylated single-chain protein with a molecular weight of approximately 38 kDa. Amb a 2 shows about 65% identity with Amb a 1, and 70% of ragweed sensitive individuals were shown to react to Amb a 2 [85, 86]. The minor allergen Amb a 3 is a basic glycoprotein and belongs to the plastocyanin family, which are copper-containing plant proteins that are involved in electron transport. About 30-50% of ragweed-sensitive patients react to Amb a 3. Amb a 7 is considered to be a plastocyanin as well and about 15-20% of ragweed sensitized individuals where shown to have specific IgE antibodies [87]. Amb a 5 is a very small basic protein, with a molecular weight of only 5 kDa. 10-20% of ragweed-hypersensitive patients display IgE against Amb a 5 [88]. The most recently characterized ragweed allergen is Amb a 4, which shows homology to Art v 1 from mugwort. More than 30% of ragweed-sensitive patients reacted to Amb a 4 [85].
Important pan-allergens contained in ragweed pollen are Amb a 6 (lipid transfer protein), Amb a 8 (profilin) as well as Amb a 9 and Amb a 10 (polcalcins). Amb a 6 is a basic protein, belonging to a group of plant nonspecific lipid transfer proteins (nsLTP). Originally, these proteins were named according to their ability of binding and transferring many different lipid molecules between membranes *in vitro*, although they don't have this function *in vivo*. Often they are located in the peal of fruits rather than in the pulp [89]. In ragweed it is a minor allergen with 21% of ragweed-sensitive patients showing reactivity to Amb a 6 [90].

Allergen	Biologic function	Molecular weight [kDa]
Amb a 1	pectate lyase	38
Amb a 2	pectate lyase	38
Amb a 3	Plastocyanin; minor allergen	11
Amb a 4		30
Amb a 5	minor allergen	5
Amb a 6	lipid transfer protein; pan-allergen	10
Amb a 7	plastoccyanin	12
Amb a 8	profilin; pan-allergen	14
Amb a 9	calcium-binding protein; pan-allergen	9
Amb a 10	calcium-binding protein; pan-allergen	

Table 1: Ragweed pollen allergens

Amb a 8 belongs to the family of profilins. Profilins share more than 75% of highly conserved sequence identities, which leads to highly similar structures and biologic functions [91]. As profilins are ubiquitously spread, they are for example responsible for allergic cross-

reactions between ragweed, melon, and banana (by Amb a 8, Cuc m 2, and Mus xp 1, respectively) [92]. Both, Amb a 9 and Amb a 10 belong to the polcalcins, a group of calcium-binding proteins that share common helix-loop-helix domains, called EF-hands. Polcalcins are specifically expressed in pollen tissues and therefore not associated with allergy to plant-derived foods [93].

1.3.3 Influence of climate change on ragweed pollen allergenicity

Climate change, characterized by increased concentrations of CO_2 and other greenhouse gases, and by warming of the earth atmosphere, is known to influence the growing conditions of plants and therefore alters their production of pollen. This can lead to increased prevalence and severity of atopic diseases, like allergic asthma.

Different studies have examined the influence of elevated concentrations of CO_2 and increased temperature on ragweed plants and pollen. Ziska at al. and Wayne et al. could show that CO_2 –enrichment leads to a significant increase in ragweed pollen production [94, 95]. Not only the amount of pollen, but also the content of the major allergen Amb a 1 is influenced by CO_2 levels. Rising CO_2 concentrations were shown to increase Amb a 1 content, showing that CO_2 directly influences ragweed pollen allergenicity. Additionally, ragweed plants, which were grown in urban areas and therefore are exposed to more CO_2, produced significantly more biomass and grew faster, then ragweed plants in rural areas [96]. Climate change is accompanied by global warming and increased temperature was shown to lead to a prolonged flowering time of ragweed as well as increased pollen production [94].

Besides changes in CO_2 levels, an increase of ozone (O_3) levels can lead to modifications that influence the allergenicity of plants. However, it was shown, that elevated concentrations of ozone affected neither total biomass of ragweed plants nor vegetative or reproductive factors [97]. In contrast, elevated ozone exposure seems to affect ragweed pollen after its dispersion in the atmosphere. Pasqualini et al. showed that ozone fumigation of ragweed pollen resulted in a significant decrease of pollen viability but also in induction of NADPH oxidase activity [98]. Elevated levels of ozone levels during growing of ragweed plants showed no influence on pollen size, shape or surface structure. Although transcriptomic analysis showed changes in in transcript levels of allergens, no influence on protein amount of the major allergen Amb a 1 under increased ozone concentrations was measurable [99]. After entering the plant cell, ozone rapidly leads to oxidative stress, by forming reactive oxygen species (ROS) by reacting with cell wall components and lipids. Therefore, even if there was no influence of increased

ozone concentration on plant growth or total protein content of pollen, it should be considered that ozone can affect the pollen maturation during growing.

1.3.4 Increase of ragweed allergy

In most industrialized countries there has been an increase of the prevalence of allergies in the last two decades [100]. Ragweed is one of the most frequent causes of allergic symptoms in Northern America, where the prevalence of ragweed allergy in atopic individuals is about 45 % and up to 15 million people suffer from symptoms of ragweed pollen allergy [101, 102]. Ragweed grew originally in Northern America and it is estimated that in the 19th century it was imported to Europe by contaminated seeds. During the last decades ragweed plants spread mainly in Hungary, Italy, France, and Austria [103]. Since the year 2005 an extensive spreading of ragweed plants and consecutive development of allergies is observed in Germany. Currently most ragweed stands are found in the eastern and southern part of Germany, especially along motorways [104]. The increased distribution of ragweed plants is attended by rising prevalence of ragweed allergy. Due to its late flowering time ragweed can be a health risk, especially for polysensitized atopic patients who suffer already from different allergies. In a study from Boehme et al. 10-17% of 10-year old children displayed ragweed specific IgE antibodies [105] and Ruëff et al. showed that 19,5% of patients with a suspected atopic disease or food allergy were sensitized to ragweed according to skin prick tests [106]. Even if the detection of a sensitization does not proof an evident allergic disease and could also be due to cross-reactivity to other plants of the asteraceae subfamily or to nutritional allergens, ragweed may be a serious health hazard, especially for patients with multiple pre-sensitizations, who may be sensitized more easily to new aeroallergens. Moreover, it should be considered that because of its late flowering season, ragweed can prolong the period of seasonal allergies.

1.4 Use of animal models in allergy research

For the current study two animal models for allergic asthma were established. As mentioned before, allergic asthma is a heterogeneous, multifactorial, and complex disease, which is influenced by a variety of factors. The development of allergic asthma includes many different aspects based on cellular [107], molecular [108], and genetic [109] mechanisms. To understand and investigate the cellular, biochemical and molecular processes, which lead to airway remodeling and inflammation, the ideal approach would be to conduct studies in

human asthmatics. Of course, performing mechanistic studies in human test persons is not acceptable due to ethical reasons. There are many different *in vitro* models available, either using standardized cell lines or cells obtained from healthy and asthmatic donors, which can be useful in clarifying many issues. These cell culture models can be helpful examples for understanding cellular responses or cell signaling [110-112]. However, all these *in vitro* models lack the complex interaction between large numbers of cells, proteins, and molecules involved in immune reactions in the lung and the whole body and are therefore not able to reflect the *in vivo* situation completely. Moreover, only animal models allow performing intervention studies and provide the opportunity to analyze the importance of certain genes for development of a disease. For that reason the only model, which can simulate the diverse environment and complicated processing taking place within the body is the animal model. Furthermore, *in vivo* models provide the possibility for investigation disease mechanisms and progression. Of course, before performing an animal experiment careful consideration is necessary and much effort should be put in choosing the right model.

Mouse models of allergic asthma offer many advantages and so they are used to date to clarify many issues. However, there are also disadvantages in using these models and many review articles address these benefits and problems [113-124].

1.4.1 Benefits of murine models

Murine models offer many benefits. First of all the breeding of mice is relatively cheap compared to other animal models and therefore allows conducting of studies with large cohorts and many different read outs. Another big advantage of murine models is that there is much information about the murine genome available and that almost all genes (99 percent) of the murine genome can be lined up with the human genome [125, 126]. Furthermore, the ability to knock out genes of interest or create transgenic mice provides deep insights into human biology and mechanisms of diseases, like cancer, obesity, diabetes and heart diseases [127]. Murine animal models are very useful for mechanistic studies, as several mediators can be modified, manipulated, or suppressed. The best known example is the discovery of the relevance of Th1 and Th2 immune responses for allergic sensitization [128] and the detection of the importance of the Th2 cytokines IL-4, IL-5, and IL-13 [129-131] in development and progression of allergic asthma and airway inflammation. Furthermore, murine models also have great advantages in testing the efficacy and toxicity of drugs. Although *in vitro* models should be used to identify the effects on cellular level, *in vivo* models are important determine the effects of a new drug in the immune and respiratory

system. There are huge numbers of preclinical studies conducted in murine animal models to evaluate the effect of compounds for treatment of respiratory diseases, which were followed by clinical studies, for example corticosteroids [132], anti-IgE [133], anti-IL-4 [134], or anti-CD4/8 [135].

Although animals do not develop allergic asthma naturally, mice can be easily sensitized to many different antigens to which they are not exposed under normal circumstances. The most popular antigen is ovalbumin (OVA) and normally mice are sensitized by intraperitoneal injection of OVA precipitated with aluminum hydroxide (alum), followed by repetitive challenges with OVA, which can be conducted by aerosol, intranasally or intratracheally. Further, there is used a number of allergens, which elicit allergic reactions in humans, for example house dust mite [136, 137], cockroach antigens [138, 139], Aspergillus fumigatus [140, 141], or ragweed [142-144]. The outcome of the antigen sensitization depends strongly on the mouse strain. So, BALB/c and C57BL/6 mice differ strongly in their Th2 versus Th1 sensitization profile, with BALB/c mice developing a much stronger Th2 response after systemic sensitization compared to C57BL/6 mice [145]. It was found out as well, that only A/J show eosinophilic inflammation after repeated intranasal OVA instillation [146]. These strain differences in response to allergen exposition represent different genetic mechanisms of asthma and can mimic the different phenotypes of asthma seen in humans.

1.4.2 Problems of murine models

It is known that the transfer of knowledge derived from animal models to human system is not always possible. There are even some examples, were results, which were observed in animal models led to real catastrophes in human patients. A terrible example is Contergan, which was shown to have sedative effects in rodents, without any harmful side effects. Unfortunately, in pregnant women it led to severe malformations of the embryos [147]. Another severe problem came up during the TGN1412 catastrophe when using anti-CD28 the first time in human probands, where it unexpectedly activated all CD28 positive cells and not preferentially regulatory T cells, as it was predicted by animal studies. This caused a “cytokine storm” followed by multiple organ failure in six healthy volunteers [148] .

Therefore, also in asthma models the first and most apparent problem of mouse models is of course the transfer to humans and human asthma. Obviously, there are huge anatomical differences between humans and mice. The morphology and the branching pattern of the murine airways differ significantly from the human. Whereas the human lung shows an almost perfect dichotomous pattern, where the daughter branches are equal in diameter,

mice show a much more monopodial branching pattern, where one of the daughter branches is larger than the other one [149]. This fact can influence the distribution of allergen and other airborne substances within the lung. There are also structurally differences in the lung, as mice have a low percentage of smooth muscle in their lungs compared to humans [150], which may alter the resistance of the lung. Furthermore, the cell types involved differ between mouse and human, as particularly for the airway epithelium in mice, eosinophils seem to be the prior outcome, which is not true for humans [151]. Another discrepancy between human and murine asthma is that human asthma often starts in childhood, influenced from genetic as well as from environmental factors [152]. However, most animal models use adult animals and it would be difficult to use juvenile animals because they reach adulthood within only few weeks.

Mice do not spontaneously suffer from any conditions, which could be compared to allergic asthma [153, 154]. Therefore, the disease always has to be induced artificial and cannot be fully compared to the allergic asthma in humans.

Another point of criticism towards murine models of asthma is that most sensitizing protocols need the use of an adjuvant. Most often aluminum hydroxide (alum) is used, which is known to promote a Th2 immune response when administered systemically together with an antigen [155]. There are other adjuvants used as well, for example heat inactivated *Bordetella pertussis* [156], ricin [157, 158], or adjuvants promoting a Th1 phenotype like Freund's complete adjuvant [159]. However, some models don't require the use of adjuvants. There were attempts to establish sensitization models via airways only using OVA [160] as well as house dust mite [136]. In models using intranasal adjuvant-free OVA, the success was restricted and highly dependent on the LPS content [161] but there were achieved good results with intranasal house dust mite models. Continuous long time exposure to house dust mite extract for several weeks led to a severe inflammation without using any adjuvants [162]. This might be due to the enzymatic activity of proteases in house dust extracts [163].

As asthma is a very complex disease, no single experimental model can cover all functional, cellular, molecular, and morphological aspects of this chronic human disease. However, animal models of asthma can mimic specific features of asthma, and extend our knowledge in the basic principles of the development of allergic asthma.

1.5 Aim of this study

The objective of this project was to analyze allergenic properties of ragweed pollen as an environmentally relevant allergen in a murine *in vivo* model.
Therefore two different models of ragweed-specific allergic airway inflammation were established. The first model was meant to reach strong and robust Th2 sensitization and ragweed-specific elicitation of allergic airway inflammation by intraperitoneal injection of ragweed extract in combination with aluminum hydroxide followed by three intranasal ragweed treatments. The second model mimics the physiological route of pollen exposure via the nasal mucosa only and does not need any artificial adjuvants. This novel sensitization protocol should induce a modest inflammation which allows analysis of proinflammatory or sensitizing properties of ragweed pollen extract.
Using these two models the following issues should be addressed:

- Does the allergenicity of pollen grown under elevated levels of ozone change in intraperitoneal sensitization? Are there differences in the sensitization or the elicitation phase of allergic asthma?
- Can ragweed specific Th2 sensitization and allergic airway inflammation be induced by only consecutive intranasal instillation without adjuvant?
- Which effects does total ragweed extract, the major allergen Amb a 1 and the protein-free fraction have in intranasal sensitization?
- Which role does pollen-derived adenosine play in intranasal sensitization?
- Does intranasal ragweed instillation influence an unrelated bystander inflammation?

2 Material

2.1 Material for in vivo and in vitro experiments

Substance	Manufacturer
Adenosine	Sigma
Adenosine Deaminase	Sigma
Imject® Alum	Pierce
Amb a 1	manufactured by Dept. of Mol Biol, University Salzburg
FCS	PAA
Glutamine	Invitrogen
Isoflurane	Baxter
Ketamine	WDT
LPS	Sigma
NEM non-essential amino acids	Invitrogen
NEM Sodium pyruvate	Invitrogen
OVA	Sigma
PBS	Invitrogen
Pen/Strep	Invitrogen
Ragweed pollen	Allergon Institute for plant pathology
RPMI1640	Invitrogen
Trypanblue	Sigma
Xylazine	aniMedica
β-Mercaptoenthanol	Sigma-Aldrich

Table 2: Substances for *in vivo* and *in vitro* experiments

Solution	Contents	Composition
Anaesthezia	100 mg ketamine 0.5% xylazin	100µl ketamine 1 ml xylazin 8,9 ml PBS
BAL medium	PBS/10% FCS	5 ml FCS 45 ml PBS
Cell counting buffer		1 ml Typanblue 9 ml PBS
R10 medium	 2 mM Glutamine 1000 U/ml Pen/Strep 10% FCS 50 µM β-Mercaptoenthanol	500 ml RPMI1640 5 ml Glutamine 5 ml Pen/Strep 50 ml FCS 500 µl β-Mercaptoenthanol
R10-complete medium	 1 mM NEM sodium pyruvate 0.1 mM NEM non-essential amino acids	R10 medium 5.6 ml NEM sodium pyruvate 5.6 ml NEM non-essential amino acids
Red blood cell lysis buffer (10x)	1.5 M NH_4Cl 10 mM $NaHCO_3$ 10 mM disodium EDTA	80.2 g NH_4Cl 8.4 g $NaHCO_3$ 3.7 g disodium EDTA q.s. to 1l/ pH 7.4

Table 3: Buffers/media for in vivo and in vitro experiments

2.2 Material for ELISA

Name	Contents		Manufacturer
Amb a 1	Capture	Rabbit anti-Amb a 1	Indoor biotechnologies
	Detection	Biotinylated rabbit anti-Amb a 1	
	Standard	Amb a 1 allergen	
Mouse IFN-γ ELISA Set	Capture	Anti-mouse IFN-γ	BD OptEIA™
	Detection	Biotinylated anti-mouse IFN-γ	
	Enzyme	Streptavidin- HRP conjugate	
	Standard	Recombinant mouse IFN-γ	
Mouse IgG ELISA Quantification Set	Capture	Goat anti-mouse IgG	Bethyl Laboratories
	Detection	Goat anti-mouse IgG-HRP	
	Standard	Mouse reference serum	
Mouse IL-10 ELISA Set	Capture	Anti-mouse IL-10	BD OptEIA™
	Detection	Biotinylated anti-mouse IL-10	
	Enzyme	Streptavidin- HRP conjugate	
	Standard	Recombinant mouse IL-10	
Mouse IL-13 Cytoset	Capture	Anti-mouse IL-13	Invitrogen
	Detection	Anti-mouse IL-13 Biotin	
	Enzyme	Streptavidin- HRP conjugate	
	Standard	Recombinant mouse IL-13	
Mouse IL-4 ELISA Set	Capture	Anti-mouse IL-4	BD OptEIA™
	Detection	Biotinylated anti-mouse IL-4	
	Enzyme	Streptavidin-HRP conjugate	
	Standard	Recombinant mouse IL-4	
Mouse IL-5 ELISA Set	Capture	Anti-mouse IL-5	BD OptEIA™
	Detection	Biotinylated anti-mouse IL-5	
	Enzyme	Streptavidin-HRP conjugate	
	Standard	Recombinant mouse IL-5	

Table 4: Commercial ELISA kits

Antibodies and proteins were used in concentrations given in the datasheets

Name	Contents		Concentration/ Dilution	Manufacturer
Total IgE	Capture	Sheep anti-mouse IgE	10 µg/ml	The Binding Site
	Detection	Biotin conjugated anti-mouse IgE	1.25 µg/ml	BD Pharmingen™
	Enzyme	Streptavidin HRP	1 µg/ml	Calbiochem
	Standard	Purified mouse IgE, κ	250-0.98 ng/ml	BD Pharmingen™
OVA IgE	Capture	OVA V	10 µg/ml	Sigma-Aldrich
	Detection	Biotin conjugated anti-mouse IgE	1.25 µg/ml	BD Pharmingen™
	Enzyme	Streptavidin HRP	1 µg/ml	Calbiochem
	Standard	Mouse Anti-ovalbumin IgE	1000-3.9 ng/ml	Biozol
OVA IgG_1	Capture	OVA V	1 µg/ml	Sigma-Aldrich
	Detection	Anti-mouse IgG_1 biotin	1.25 µg/ml	Pharmingen™
	Enzyme	Streptavidin HRP	1 µg/ml	Calbiochem
	Standard	Anti-chicken egg albumin clone OVA 14	250-0.49 ng/ml	Sigma-Aldrich
Amb a 1 IgE	Capture	Amb a 1	See results	University Salzburg
	Detection	Biotin conjugated anti-mouse IgE	1.25 µg/ml	BD Pharmingen™
	Enzyme	Streptavidin HRP	1 µg/ml	Calbiochem
	Standard	Serum from sensitized mice	1:5 – 1:5120	own laboratory
Amb a 1 IgE	Capture	FcεRI	1:100	
	Detection	Biotinylated Amb a 1	See results	own laboratory
	Enzyme	Streptavidin HRP	1 µg/ml	Calbiochem
	Standard	Serum from sensitized mice	1:5 – 1:5120	own laboratory
Amb a 1 IgG_1	Capture	Amb a 1	See results	Salzburg
	Detection	Anti-mouse IgG_1 biotin	1.25 µg/ml	BD Pharmingen™
	Enzyme	Streptavidin HRP	1 µg/ml	Calbiochem

	Standard	Serum from sensitized mice	1:500 – 1:512000	own laboratory
Amb a 1 IgG_{2a}	Capture	Amb a 1	See results	Salzburg
	Detection	Anti-mouse IgG_{2a} biotin	1.25 µg/ml	BD Pharmingen™
	Enzyme	Streptavidin HRP	1 µg/ml	Calbiochem
	Standard	Serum from sensitized mice	1:500 – 1:512000	own laboratory

Table 5: Self-designed ELISAs

Chemical	Forumla	Manufacturer
BSA		Sigma-Aldrich
Citric acid monohydrate, ACS reagent	$HOC(COOH)(CH_2COOH)_2 \bullet H_2O$	Sigma-Aldrich
Dimethylsulfoxide (DMSO)	$(CH_3)_2SO$	Merck
Disodium carbonate	Na_2CO_3	Sigma-Aldrich
Ethanol	C_2H_5OH	Merck
FCS		PAA
Hydrochloric acid	HCL	Merck
Hydrogen peroxide	H_2O_2	Sigma-Aldrich
Potassium chloride	KCl	Merck
Potassium hydrogen phosphate	KH_2PO_4	Merck
Potassium hydroxide	KOH	Sigma-Aldrich
Sodium chloride	NaCl	Sigma-Aldrich
Sodium hydrogen carbonate	$NaHCO_3$	Merck
Sodium phosphate dibasic	Na_2HPO_4	Sigma-Aldrich
Sulfuric acid	H_2SO_4	Merck
Tetramethylbenzidin (TMB)	$C_{16}H_{20}N_2$	Fluka
TRISma®base	$NH_2C(CH_2OH)_3$	Sigma-Aldrich
Tween®20	$C_{58}H_{114}O_{26}$	Sigma-Aldrich

Table 6: Reagents/chemicals for ELISA buffers

Solution	Contents	Composition
Assay diluent	1xPBS/10%FCS	50 ml 10xPBS 50 ml FCS 400 ml aqua dest.
Blocking buffer	1xTris/1% BSA	5 g BSA 50 ml 10xTris 400 ml aqua dest.
Coating buffer	0.1 M sodium carbonate	8.4 $NaHCO_3$ 3.56 Na_2CO_3 q.s. to 1 l in aqua dest. / pH 9.5
Developing reagent	TMB buffer	55 µl TMB stock 2,55 µl H_2O_2 5.5 ml substrate buffer
PBS (10x)		80 g NaCl 11,6 g Na_2HPO_4 2 g KH_2PO_4 2 g KCL q.s. to 1 l in aqua dest./ pH 7.4

Stop solution	2 N sulphuric acid	30 ml H_2SO_4 180 ml aqua dest.
Substrate buffer	0.2 M citrate buffer	42.05 g citric acid monohydrate q.s. to 1 l/ pH 3.95
TMB stock		24 TMB 500 µl Ethanol 500 µl DMSP
TRIS-buffer (10x)	500 mM TRISma®base	60.55 g TRISma®base q.s. to 1 l in aqua dest. pH t.4
Washing buffer	PBS/0.05% Tween	100 ml 10xPBS 900 ml aqua dest. 0,5 ml Tween20
	Tris/0.05%Tween	100 ml 10xTris 900 ml aqua dest. 0,5 ml Tween20

Table 7: Buffers for ELISA

2.3 Material for histology

Solution	Formula	Manufacturer
Entellan®		Merck
Eosin Y, alcoholic	$C_{20}H_6Br_4Na_2O_5$	Thermo Scientific
Ethanol absolute		Hemalm
Formaldehyde	CH_2O	Staub&Co
Hematoxylin	$C_{16}H_{14}O_6$	Merck
Paraffin		McCormick™ Sc.
Periodic Acid		Merck
Pertex		Medite
Schiff's reagent		Merck
Xylene		Hedinger

Table 8: Buffers/Solutions for hisotolgy

2.4 Commercial kits

Kit	Components	Manufacturer
Diff Quick staining set	Fixation solution: Fast Green in methanol Stain solution I:Eosin in phosphate buffer Stain solution II:Thiazin-dye in phosphate buffer	Medion Diagnositc
EZ-Link NHS-PEG4-Biotinylation Kit	EZ-Link NHS-PEG 4 Biotin PBS Pack Desalting Columns HABA solution Avidin	Thermo scientific
Limulus Amebocyte Lysate (LAL)	Lysate Endotoxin LAL Reagent Water	Lonza
PAS staining kit	Periodic acid 0.5% Schiff's reagent	Merck
RevertAid First Strand cDNA Synthesis Kit	RevertAid M-MuLV Transcriptase RiboLock RNase Inhibitor 5x Reaction Buffer 10 mM dNTP Mix Oligo(dT)$_{18}$ Primer	Thermo Scientific

	Random Hexamer Pimer Forward GAPDH Primer Reverse GAPDH Primer Control GAPDH RNA Water, nuclease free	
RNA Kit	RNeasey Mini Spin Columns Collection Tubes (1.5/2 ml) Buffer RLT Buffer RW1 Buffer RPE RNase-Free Water	Qiagen
RNase-Free DNase Set	DNase I, RNase-free Buffer RDD RNase-Free Water	Qiagen
SYBR Select Master Mix		Applied Biosystems

Table 9: Kits

2.5 Primers for qPCR

Primer name	Sequence 5'-3'		Manufacturer
IL-4	Forward: Reverse:	TGA ACG AGG TCA CAG GAG AA CGA GCT CAC TCT CTG TGG TG	Metabion
IL-13	Forward: Reverse:	TGT GTC TCT CCC TCT GAC CC CAC ACT CCA TAC CAT GCT GC	Metabion
IFN-gamma	Forward: Reverse:	CAC GGC ACA GTC ATT GAA AG GCT GAT GGC CTG ATT GTC TT	Metabion
GM-CSF	Forward: Reverse:	CAT CAA AGA AGC CCT GAA CC CGA ATA TCT TCA GGC GGG T	Metabion
TNF-alpha	Forward: Reverse	CCA CCA CGC TCT TCT GTC TAC AGG GTC TGG GCC ATA GAA CT	Metabion
IL-1beta	Forward: Reverse:	TGA AGC AGC TAT GGC AAC TG AGG TCA AAG GTT TGG AAG CA	Metabion
IL-17	Forward: Reverse:	ACT ACC TCA ACC GTT CCA CG AGA ATT CAT GTG GTG GTC CAG	Metabion
Muc5ac	Forward: Reverse:	TGG AGT CAG CAC GAA AAC AG GCA CTG GGA AGT CAG TGT CA	Metabion
IL-5	Forward: Reverse:	AGC AGT GGT GAA AGA GAC CTT TCA ATG CAT AGC TGG TGA TTT	Metabion
IL-22	Forward: Reverse	GCT CAG CTC CTG TCA CAT CA TCG CCT TGA TCT CTC CAC TC	Metabion
IL-6	Forward: Reverse	GAT GGA TGC TAC CAA ACT GGA TCT GAA GGA CTC TGG CTT TG	Metabion
MIP-2	Forward: Reverse	CCT GGT TCA GAA AAT CAT CCA CAT CAG GTA CGA TCC AGG CT	Metabion
KC	Forward: Reverse:	CCA CAC TCA AGA ATG GTC GC TCT CCG TTA CTT GGG GAC AC	Metabion
IL-10	Forward: Reverse:	ATC GAT TTC TCC CCT GTG AA TGT CAA ATT CAT TCA TGG CCT	Metabion
IL-33	Forward: Reverse:	TGC GTC TGT TGA CAC ATT GA GAC TTG CAG GAC AGG GAG AC	Metabion
Arg 1	Forward: Reverse:	AGA GAT TAT CGG AGC GCC TT TTT TTC CAG CAG ACC AGC TT	Metabion
Muc2	Forward: Reverse:	TGA TGC CAA TGA CAA GGT GT AGC CTG GAG ATG TTC ACC AC	Metabion
GAPDH	Forward: Reverse	CGT CCC GTA GAC AAA ATG GT TTG ATG GCA ACA ATC TCC AC	Metabion

Table 10: Primers for qPCR

2.6 Consumables

Material	Type	Manufacturer
96 well plate	Cell culture	Nunclon™Δ
	Maxisorp	Nunclon™Δ
Canula	Serican G17, G20	Braun
Cell strainer	70 µm	BD Falcon
Coverslip	24x60 mm	Menzel-Glaser
FACS tubes	Microtubes	Alpha laboratoires
Glass slide	50 Elka	Hecht-assistant
Heparin tubes	LI 1000 A	KABE Labortechink
PCR tubes		Eppendorf
Pipettes	1 ml	Greiner bio-one
	2 ml	Greiner bio-one
	5 ml	Greiner bio-one
	10 ml	Greiner bio-one
	25 ml	Greiner bio-one
Safelock tubes	0.5 ml	Eppendorf
	1,5 ml	Eppendorf
	2.0 ml	Eppendorf
Sterile filter	Millex 0.22 µm	Millipore
	Filtropur V50.02	Sarstedt
Syringes	Omnifix®(1, 5, 10, 20 ml)	Braun
Tips	10 µl	Eppendorf
	200 µl	Eppendorf
	300 µl	Eppendorf
	1000 µl	Eppendorf
Tissue embedding cassette		Simport
Tubes	15	Sarstedt
	50 ml	Sarstedt

Table 11: Consumables

2.7 Technical and laboratory equipment

Equipment	Type	Manufacturer
Cell counter	Cellometer™ Auto T4	Thermo Fischer
Centrifuge	Magafuge1.0R	Heraeus
	Laborfuge 40R	Heraeus
	Biofuge pico	Heraeus
CO_2 incubator	IR-Sensor	Sanyo
Compressor		600-4 BS
Cyto centrifuge	Cytospin3	Shandon
Drying chamber	UT 6060	Haraeus
Embedding machine	EG 1150C	Leica Co
Freezer (-20°C)	GS 5203-11	Liebherr
Freezer (-80°C)	KLT 4785	Kryosafe
Fridge (4°C)	UKS 500-10	Liebherr
Homogenizer	TissueLyser LT	Qiagen
Hood	JS 15	Heraeus
Immunostainer	TechMate Horizon Automated Immunostainer	Dako
Micotome	HM355S	Microm
Microscope	Axiovert 40CFL	Zeiss
	MS 5, Stereomicroscope	Leica
	Leica DM LB	Leica
Nebulizer		Pariboy
Neubaur counting chamber	Haemacytometer- CE doppelt	Assistent
pH meter	CG 841	Schott
Photometer	Multiscan Ascent V1.24	Thermo Fischer

	Epoch	BioTek
Pipettes	Reference (10, 20, 100, 200, 1000 µl)	Eppendorf
	Multipette	Eppendorf
	Morechanel	Eppendorf
Pipettor	Accu-jet	Brand
Real-Time PCR System	ViiA™7	Applied Biosystems
Sonication bath	Sonorex R52	Bandelin
Thermocycler	Peqstar	Peqlab Biotechnologie GmbH
Tissue drying oven	TDO50	medite
Tissue processor		Shadon Co.
Tissue strainer	COT	medite
Vortex		MS 1 Minishaker
Washer for microttiterplates	Hydrospeed	Tecan
Water bath	1083	GFL
	PSI	Medizintechnik Grünwald
Water purification device	MilliQ 1, EASYpure UV	Millipore

Table 12: Technical devices and laboratory equipment

2.8 Software

Software
Ascent Software, Version 2.6, Thermo Labsytems (ELISA reader)
Cellometer Auto T4, Version 3.3.3, Nexolon Bioscience LLC (Cell counter)
Endnote® X5, Thomson Reuters (Literature database)
Gen5, BioTek Instruments (Epoch ELISA reader)
Graph Pad Prism 6, GraphPad Software (Biostatistics, curve fitting)
Leica IM1000, Leica (Microscope)
Microsoft Office, Professional Edition 2003, Microsoft Corporation
Nanodrop® 100 V 3.7.0
Adobe Photoshop CS5 Extended, Adobe

Table 13: Software

3 Methods

3.1 Pollen preparation

3.1.1 Cultivation of ragweed plants

Ragweeds plants were seeded and grown by our cooperation partners from the Institute for Plantpathology at the Helmholtz Zentrum München. In short, seeds from a single plant were applied to standard soil in pots and plants were cultivated in Plexiglass sub-chambers placed within two phytotron walk-in chambers [164]. The light regime, temperature and humidity were adapted to the seasonal cycle, starting with the climate conditions of May 2010. The light period was 14.5 h d^{-1}; day/night temperatures were 20-30 °C/ 10-20 °C and relative humidities were 80-85%/30-50% (day/night). Watering was carried out by an automated watering system. The plants were fumigated with 40 bbp (control) and 80 ppb ozone for the whole vegetation period. Pollen were collected from July to August, using a modified ARACON system (BETATECH, Ghent, Belgium) that covered the male inflorescenses [99]. Pollen samples were stored at -80°C until use.

3.1.2 Preparation of pollen extracts and extract fractions

Aqueous pollen extracts were generated by incubating 10 mg of pollen in 1 ml of sterile PBS for 30 minutes at 37°C with vortexing every ten minutes. After centrifuging for ten minutes at 3000 g the supernatant was passed through a 0.2 µm sterile filter, aliquoted and stored at -80°C.

For fractionating the pollen extracts based on molecular size, the extracts were passed three times over a 3 kDa cutoff filter, generating a protein-free fraction (RWE <3 kDa), which includes PALMs and other low-molecular weight substances, and a protein-containing fraction (RWE >3 kDa). To remove adenosine, RWE were digested with 100 U/ml adenosine deaminase (ADA) for 1 hour at 25°C. The enzyme was removed by means of ultrafiltration with a 3 kDa cutoff filter. The absence of proteins in the RWE <3 kDa was verified by Coomassie Protein Assay.

3.1.3 Determination of protein content by coomassie assay in pollen extracts

After diluting the standard according to the manufacturer's instructions, 10 µl of samples and standards are added into a 96 well plate, followed by 200 µl of Coomassie Plus Protein Assay Reagent and shaked softly for 10 sec. After 10 min incubation at room temperature, the absorbance is measured at 595 nm.

3.1.4 Determination of endotoxin by LAL assay in pollen extracts

LPS (endotoxin) was quantified by Limulus Amebocyte Lysate (LAL) assay according to the manufacturer. In brief, plates a pre-equilibrated to 37°C ± 1°C in a heating block adapter, and then 50 µl of sample or standard were added into the appropriate well. Each series of determination included a blank (containing 50 µl of LAL reagent water) plus for endotoxin standards run in duplicate. Fifty microliter of LAL were added for 10 min at 37°C, than 100 µl of substrate solution (prewarmed to 37°C) was added for 6 min, finally 100 µl of stop reagent was added and the absorbance was measured at 405 nm.

3.1.5 Measurement of the major allergen Amb a 1

For detection of Amb a 1 in the ragweed extract (RWE) an Indoor Biotechnologies kit was used with slight modifications. In brief, plates were coated with 100 µl of a 1:1000 dilution of the coating antibody overnight and blocked with 300 µl of PBS/1%BSA for 30 min. After incubating 100 µl of standards (0,25-0,0005 µg/ml) and samples (dilution 1:500 – 1:8000) for one hour, a biotinylated rabbit anti-Amb a 1 detection antibody is added for one hour and detected via streptavidin-peroxidase. Thereafter, substrate buffer is added and the reaction is stopped by sulfuric acid. Plates are read at 450 nm.

3.1.6 Biotinylation of Amb a 1 or total RWE

The biotinylation of Amb a 1 was done according to the manufacturer's instructions. The principle of the biotinylation is based on the reaction of the primary amino groups ($-NH_2$) of proteins with NHS-PEG_4-Biotin by nucleophilic attack. Thereby, a stable amine bond is formed between the primary amine and the PGE_4-Biotinm whereas the NHS group is released. The leaving NHS group and the non-reacted biotin reagent are removed afterwards by a desalting step. In brief, Amb a 1 solution was added to an appropriate volume of NHS-PEG_4-Biotin solution, calculated according to the manufacturer's instructions with 38 kDa as

molecular weight of Amb a 1. After incubation at room temperature for 60 minutes, excess biotin regent was removed using a desalting column. As the total RWE consist of many different proteins and also other substances, which may carry an amino group, for calculation of the right volume of NHS-PEG_4-Biotin solution an average molecular weight of 17 kDa was assumed.

3.2 Animal handling

Female BALB/c mice were obtained from Charles River (Sulzfeld/Germany) and housed under specific pathogen free (SPF) conditions in individually ventilated cages (VentiRack; Biozone, Margate/UK) and fed by standard diet and water ad libitum. The study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Center Munich.

Intraperitoneal injection: I.p. injection was done in the lower left quadrant of the abdomen. Not more than 200 µl were injected with moderate pressure and speed.

Intranasal instillation: Anesthetized mice were instilled with 10 µl liquid per nare using a 10µl pipet.

Anesthesia of mice: To anesthetize mice for up to 30 min. i.p. injection of a 10% Ketamine/2% Xylazin mixture in PBS was used. One hundred microliters per 10 g bodyweight were administered. For short term anesthesia of mice isofluoran inhalation was used.

Euthanasia: Mice were euthanized by cervical dislocation. Mice used for BAL analysis were given an overdose administration of injectable anesthetic, followed by death without regaining consciousness.

Blood collection: Blood collection was done by puncture of the orbital sinus or plexus of mice. About 200 µl was collected from anesthetized mice in two weeks intervals from alternate sides. For recovery mice were administered 200 ml of sterile PBS. Blood was sampled in heparinized tubes and then centrifuged for 7 min at 5000 rpm. Plasma was transferred in a new tube and stored at -20°C for further analysis.

3.3 Sensitization protocols

3.3.1 Intraperitoneal sensitization against ragweed pollen extract (RWE)

For intraperitoneal sensitization female 6 weeks old BALC/c mice were intraperitoneal injected with ragweed extract in combination with alum (2 mg per mouse) in different doses and at different time points, to find the right concentration for an optimal sensitization. For elicitation of the allergic airway inflammation, mice were challenged intranasally on 3 days with 2 days break in between. Control mice were injected i.p. with PBS/alum and instilled intranasally with PBS.

3.3.2 Intranasal sensitization against ragweed pollen extract (RWE)

For intranasal sensitization anesthetized female 10 weeks old BALB/c mice were treated with 10 µl of RWE per nare on 11 consecutive days. Some mice were intranasally boosted on day 26, 27, and 28 as well as on day 41, 42, and 43. Control mice were treated with PBS.

3.3.3 Evaluation of bystander effect of intranasal ragweed pollen extract on OVA-sensitization

For evaluation of the bystander effect of intranasal RWE on an existing OVA-sensitization, mice were OVA-sensitized by injection of 1 µg OVA, 2 mg alum in 200 µl PBS on day 0, 7, 14, 28, and 42. Non-sensitized mice received intraperitoneal injection of PBS. After the OVA-sensitization phase, mice were instilled on 11 consecutive days with RWE. On the last day of RWE-instillation, mice underwent OVA-challenge by nebulation of 12 ml 1% OVA in PBS for 20 minutes in a chamber (14.5x23x22 cm). This protocol induces a very mild OVA specific airway inflammation, which allows us to study the additional effects of intranasal RWE treatment.

3.4 Bronchoalveolar Lavage (BAL) and total cell count

After euthanization, the chest was opened, the trachea was cannulated, and BAL was performed. In brief, lungs were rinsed five times with 0.8 ml PBS. The thorax of the mouse was massaged and the PBS was harvested by gentle aspiration. The BAL fluid (BALF) of the

first aspiration was collected separately and after centrifugation at 1000 rpm for 10 min supernatants were stored at -80°C. Cells from all five rinses were pooled, centrifuged, and resuspended in 0.5 ml RPMI/5%FCS for quantification in a 1:2 dilution in trypan blue. All four quadrants were counted and final cell yield was done with the average cell yield of two counts: Cell yield = average count/4 * volume * dilution factor * 10^4.

BAL cells were further used for cytospin preparation.

3.5 Cytospins and differential cell count

At least two cytospins were prepared from each sample of the BAL cells. About 50 000 cells were spun onto slides with a cytospin centrifuge in a total volume of 150 µl at 40 rpm for 10 min at an acceleration "medium". After drying, slides were fixed and stained with Diff-Quick staining set, according to the manufacturer's instructions. In brief, cytospins are dipped five times for one second in fixative solution, stain solution I and stain solution II and after drying mounted in Entelan. At least 400 leukocytes per sample were counted and by using standard morphological criteria distinguished in macrophages, lymphocytes, eosinophil, and neutrophil granulocytes.

3.6 Lung preparation and sectioning

After BAL, lungs were excised and one lobe was frozen in liquid nitrogen, the other lobe was fixed for histophathological examination in 4% formalin. Fixed lungs were sectioned sagittally and dehydrated through increasing concentrations of ethyl alcohol (70%, 80%. 90%, and 100%), cleared in xylene, infiltrated in paraffin by an automatic tissue processor, and embedded in paraffin wax. Four micrometer thick tissue sections were cut from the paraffin blocks with a microtome and were placed on a glass slide, which were placed for 30 min in 65°C, so the paraffin melts and the tissue is bond onto the glass.

3.7 Staining of lung sections

3.7.1 **Hematoxylin and Eosin: H&E Staining**

For staining with hematoxylin, slides were left in hematoxylin solution for 3x2 min, rinsed first with deionized water and then with tap water for 2 min. After removing an excess of

water, slides were left in eosin for 2 min, followed by dehydration in 100% ethanol with 3x2 min. Afterwards slides were covered with coverslips using mounting medium.

3.7.2 Periodic Acid Schiff's: PAS Staining

For PAS-Staining, deparaffined tissue sections were first left in periodic acid solution for 5 min and then carefully rinsed with distilled water. Then, slides were placed in Schiff's reagent for 15 min, washed in tap water for 10 min, counterstained in Mayer's hematoxylin for 5 min, and again washed in tap water for 5 min. After dehydrating slides in ascending alcohol series (5 min in 70% ethanol, 5 min in 96% ethanol, 5 min in 100% ethanol), slides were cleared with xylene and finally coverslips were mounted.

3.8 Gene expression analysis

3.8.1 Isolation of RNA

Organs for RNA isolation (lung, spleen, lymph nodes, lives, bone marrow) were directly after the BAL frozen in liquid nitrogen and afterwards stored at -80°C for further use.
Total RNA was isolated using a Qiagen RNeasy mini kit the according to the manufacturer's instructions. In brief, frozen tissues were homogenized on ice in RLT buffer, centrifuged to remove the detritus and ethanol was added to the supernatant. After transferring the supernatant in the RNeasy spin column, the total RNA binds to the membrane. The column was rinsed with RW1 buffer, followed by an on-column DNase digestion for 15 min. Afterwards the column was washed again with RW1 buffer once and with PPE buffer twice, to remove all contaminates. Afterwards the total RNA was eluted with 30 µl RNase-free water and the concentration is measured at nanodrop. The samples can be stored at -80°C

3.8.2 Generation of cDNA

Complementary DNA (cDNA) was prepared by reverse transcription using the RevertedAid H Minus First Strand cDNA synthesis kit (Fermentas GmbH/Germany) according to the manufactures instructions. In brief, 0.5 µg of total RNA were mixed with Oligo$(dT)_{18}$ Primers and RNase-free water and denatured 5 min at 65°C. In the meanwhile a master mix containing reaction buffer, RNase Inhibitor, dNTP, and reverse transcriptase is prepared and

dispensed into tubes with RNA and primers. Then, samples are incubates at 42°C for 60 min. The reaction is stopped by incubating samples for 5 min at 70°C. Samples can be stored at 4°C for short time, long time storage is at -20°C.

3.8.3 Quantitative real time PCR

Quantitative real-time PCR (qPCR) was conducted using SYBR green master mix and an ABI ViiA™ 7 System thermocycler (Applied Biosystems). 40ng of cDNA and 0.2 µM primers were used. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as housekeeping gene.

3.9 Cell culture

All cell culture experiments were conducted under sterile conditions. Centrifugations were performed at 1200 rpm (~300 g) for 10 min at 4°C. After centrifugation, the supernatant was aspirated and the cell pellet softly resuspended in appropriate buffer. All cells were incubated at 37°C in 5% CO_2.

3.9.1 Isolation of cells from spleen and lymph nodes

For gaining of splenocytes spleens were dissected under sterile conditions. After cutting into smaller pieces, the spleen was squeezed through a 70 µm cell strainer with RPMIcomplete to yield a single cell suspension. After centrifugation, the cells are resuspended in 10 ml lysis buffer for 5 min. The reaction is stopped with 10 ml RPMIcomplete and cells are centrifuged and resuspended in 10 ml RPMIcomplete.
Cervical lymph nodes were passed through a 70 µm cell strainer with RPMIcomplete, centrifuged and resuspended in 5 ml RPMIcomplete.

3.9.2 Restimulation of cells

Lymph node and spleen cells were resuspended at a density of $2x10^5$ cells per well in RPMIcomplete and restimulated with either OVA (5 µg/ml), Amb a 1 (5 µg/ml), or RWE (1.25 mg/ml) in 96 well round-bottom plates. After 5 days supernatants were collected and IL-4, IL-5, IL-13, IL-10 and IFN-y were measured by ELISA.

3.10 Enzyme-linked immunosorbent assay (ELISA)

All ELISAs were run in 96-well Micro Well™ Maxi Sorp flat bottom plates, with a standard curve on every plate. For incubation plates were sealed to avoid evaporation. For washing an automatic washing system was used. The optical density (OD) was measured at 450 nm with a Multiskan Ascent 1.24 with Ascent Software or with an Epoch and Gen5 2.00 Software.

3.10.1 **Cytokine ELISA**

For detection of cytokines in BALF or cell culture supernatant BD OptEIA™ or Biosource kits were used (as listed in Table 4) according to the manufacturer's instructions with slight modifications. In brief, coating antibody was diluted as recommended by the manufacturer in coating buffer and wells were coated with 50 µl overnight at 4°C. After washing three times with PBS/tween, plates were blocked with 300 µl of assay diluent per well for 1-2 hours, to avoid unspecific binding. After washing again, 50 µl of standards samples and controls were added and incubated for 2 hours at room temperature. Then, 50 µl of appropriate biotinylated detection antibody and streptavidin-HRP were added in indicated dilutions and incubated for one hour at room temperature, followed by eight washes. Fifty microliter per well of TMB buffer were added and incubated at room temperature in the dark until color development was visible. Color development was stopped by adding 1 N sulfuric acid.

3.10.2 **Immunoglobulin ELISA**

Total IgE

Plates were coated with 50 µl of coating antibody diluted in coating buffer. After either two hours incubation at room temperature or overnight at 4°C, plates were washed with Tris/Tween and blocked with 300 µl of Tris/1%BSA for 2 hours at room temperature. After washing, 50 µl of standards, blanks, or samples were added in duplicates and incubated either over night at 4°C or for two hours at room temperature. Then, plates were washed five times. Afterwards, 50 µl of biotin-conjugated rat anti-mouse IgE diluted in Tris/1%BSA were added and incubated for two hours at room temperature. After washing, plates were incubated with 50 µl of streptavidin-horseradish peroxidase diluted in Tris/1% BSA and

incubated for 30 minutes. Subsequently, plates were washed eight times and developed by using 50 ml TMB buffer per well and incubated in the dark for five to ten minutes, until a coloration of the lowest standard was visible. Color development was stopped with 50 µl of 1 N sulfuric acid.

OVA-specific IgE and IgG_1

Plates were coated with 100 µl of OVA V diluted in coating buffer either two hours at room temperature or overnight at 4°C. After washing plates three times with Tris/Tween, plates were blocked with 300 µl of Tris/1%BSA. Plates were washed again, and 50 µ of standards, blanks, and samples were added per well in duplicates and incubated for 2 hours at room temperature or overnight at 4°C. After washing, 50 µl of biotin-conjugated anti-mouse IgE or biotin-conjugated anti-mouse IgG1 diluted in Tris/1%BSA were added and incubated at room temperature for two hours. After washing the plates, color development with TMB buffer was done as described above.

3.11 Data analysis and statistics

Calculation of data was done with Microsoft Excel or GraphPad Prism. Data are shown either as bars indicating arithmetic means with standard error or as boxplots indicating minimum, 25% percentile, median, 75% percentile, and maximum. Statistical significance was determined by Mann-Whitney U test and results were considered significant as $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

4 Results

4.1 Analysis of ragweed pollen extracts

The different ragweed pollen extracts used for the following sensitization models were analyzed for total protein content, Amb a 1 content, and LPS.

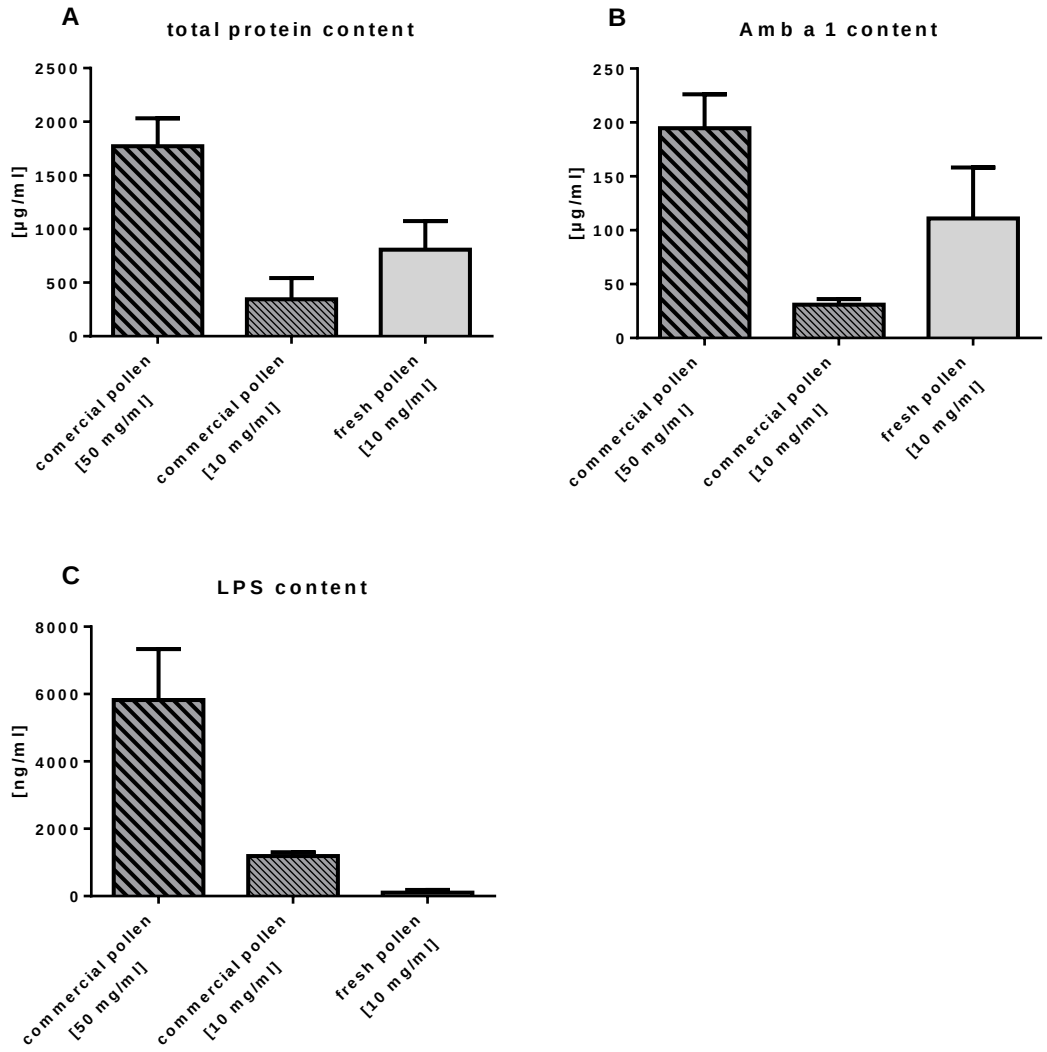

Figure 6: Determination of total protein (A), Amb a 1 content (B), and endotoxin content (C) in RWE

We used ragweed extract made of commercial ragweed pollen bought from Allergon (Sweden) in two concentrations (10 and 50 mg pollen per ml PBS) and made of freshly harvested ragweed pollen (10 mg pollen per ml PBS), which was grown at the Institute for Plant Pathology (Helmholtz Zentrum München). The total protein content as well as the content of the major protein Amb a 1 was a bit higher in the extract of freshly harvested

pollen (Figure 6A/B). Amb a 1 was about 11% respectively 9% from total protein in the commercial pollen extract (50 mg/ml respectively 10 mg/ml) and about 14% in the extract from freshly harvested pollen (Table 14). LPS content was at a high level in the extracts from commercial pollen (5822 pg/ml and 1190 pg/ml, respectively), but was almost under the detection limit in the extract from freshly harvested pollen (Figure 6C).

	Commercial pollen extract [50 mg/ml]	Commercial pollen extract [10 mg/ml]	Freshly harvested pollen [10 mg/ml]
Total protein content [µg/ml]	1772.12	344.31	806.88
Amb a 1 [µg/ml]	197.67	30.74	111.00
% Amb a 1 of total protein	10.98	8.96	13.76
LPS [pg/ml]	5821.86	1190.00	105.99

Table 14: Determination of total protein, Amb a 1 content, and endotoxin content in RWE

4.2 Establishment of a ragweed-specific ELISA-system

Specific immunoglobulins are important parameters in evaluation of allergic diseases. To detect ragweed specific immunoglobulins, we established a ragweed-specific ELISA-system, which is not commercially available. For all following experiments flat bottom 96 well maxisorp plates and a pipetting volume of 50 µl per well was used. All samples were run in duplicates.

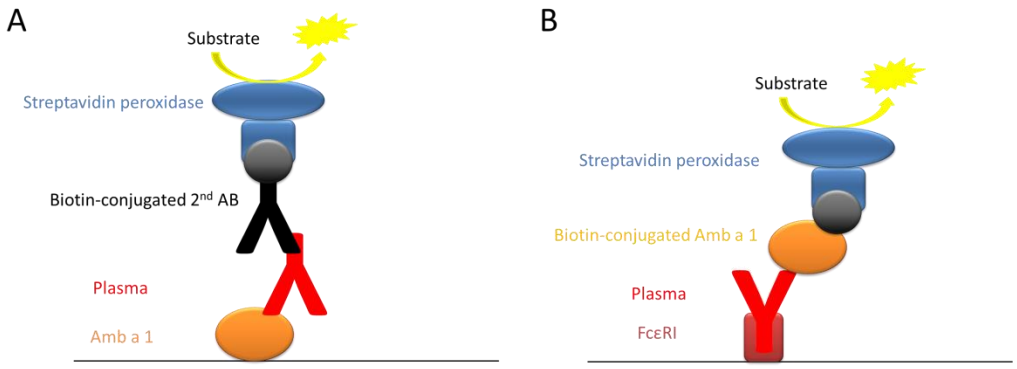

Figure 7: Set-up of ELISA systems for detections of Amb a 1 specific immunoglobulins
Sandwich ELISA to detect specific antibodies with **A**) coating of Amb a 1 and **B)** coating with the high affinity receptor for IgE (FcεRI)

The first approach to detect Amb a 1 specific IgG_1 or IgE was a modification of our established, indirect ELISA for detection of OVA specific IgG_1 and IgE (Figure 7A). For first tests, plates were coated with different concentrations of Amb a 1 (4 µg/ml, 2 µg/ml, 1µg/ml) for two hours at room temperature. To exclude an unspecific binding of the plasma to either proteins or the plate itself, plates were also coated with Bet v 1 or left completely uncoated. After blocking the plates for two hours with Tris/1%BSA, plasma of either ragweed-sensitized mice (as described later in section 4.3), non-sensitized mice, or OVA-sensitized mice was added and incubated for two hours at room temperature. All following steps were done according to the established protocols for OVA specific IgE and IgG_1. In brief, a biotinylated anti-IgE or anti-IgG_1 antibody respectively was added for 2 hours, followed by streptavidin-peroxidase. For detection tetramethylbenzidin (TMB) was used and the reaction was stopped by 2M sulfuric acid. Between every step plates were washed carefully at least 5 times with Tris/Tween puffer. Different combinations for incubation times (2h, overnight) and temperatures (room temperature, 4°C, 36°C) for coating were tested but there was no difference seen in sensitivity or specificity of the ELISA. As Figure 8A shows, 2 µg/ml Amb a 1 seem to be a sufficient coating concentration for the measurement of Amb a 1 specific IgE. For IgG_1 even the lowest coating concentration of 1 µg/ml was adequate (Figure 8B).
The binding of the immunoglobulins was specific, as there was no signal in Bet v 1 coated or uncoated wells. The best dilution of plasma for measuring Amb a 1 specific IgE was 1:10 and for specific IgG_1 1:1000.

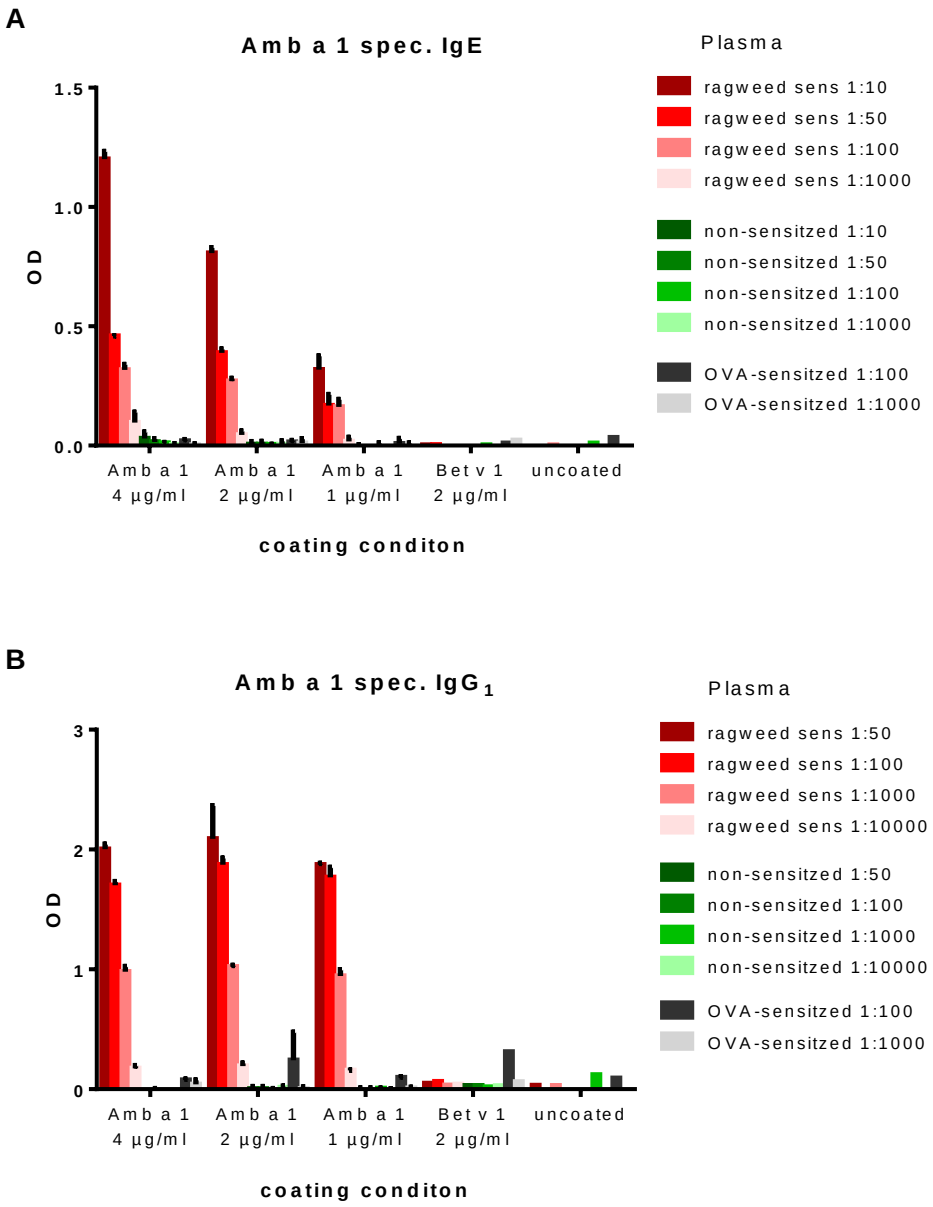

Figure 8: Establishment Amb a 1 specific ELISA
Plates were coated with different concentrations of Amb a 1 or with Bet v 1. After blocking, plasma was added in different dilutions. For analysis the specific blank was subtracted from every sample.

It is important to consider, that the plasma was taken from mice sensitized i.p. in combination with alum, which leads to very high immunoglobulin levels in plasma. In mice sensitized intranasally, immunoglobulin levels in plasma are much lower (as described later in section 4.5). Therefore using this ELISA system, only Amb a 1 specific IgG_1 was detectable but not specific IgE. One reason for this could be, that all coated Amb a 1 molecules are occupied by Amb a 1 specific IgG_1 molecules, thereby displacing IgE molecules, as discussed later. Therefore, coating with the high affinity receptor for IgE -(FcεRI) instead of Amb a 1 - was

tested. FcεRI binds all IgE molecules present in the plasma very efficiently (Figure 7B). First, plates were coated with FcεRI (1µg/ml). After addition of the plasma samples of non-sensitized, OVA-sensitized and ragweed-sensitized (intraperitoneal and intranasal) mice, a biotinylated Amb a 1 was added, which binds to the ragweed specific parts of IgE molecules. The development and quantification was carried out in the same way as described before. Not to waste Amb a 1, we tested the principle first with total RWE (Figure 9A). RWE was biotinylated with a commercial kit and added in two dilutions (1:50, 1:100) and for control biotinylated OVA was used. When using biotinylated RWE we got no accurately defined signal, which leads to the assumption that the biotinylation of the total RWE did allow specific signal detection. However, for OVA-specific IgE in plasma of OVA-sensitized mice there was a clear signal, which proofs the principle.

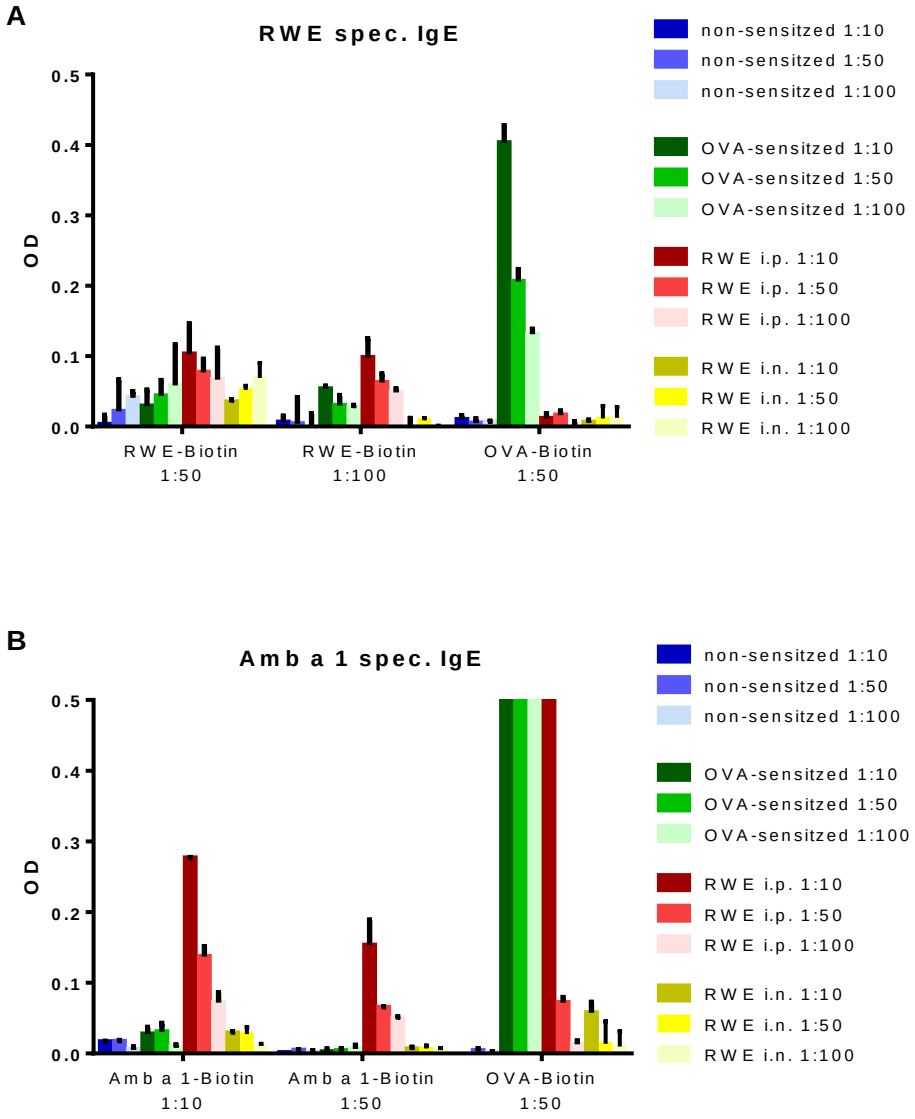

Figure 9: ELISA for ragweed specific IgE using biotinylated RWE or Amb a 1

In a next step, biotinylated Amb a 1 was used in two concentrations (1:10 and 1:50, which corresponds approximately to the concentration of Amb a 1 in the total RWE) (Figure 9B). In the plasma of i.p. sensitized animals there was Amb a 1 specific IgE detectable, but again in i.n. sensitized mice allergen specific IgE was under the limit of detection.

4.3 Establishment of intraperitoneal sensitization in combination with alum as adjuvant

To evaluate the allergenicity of ragweed pollen, we established an intraperitoneal sensitization model in combination with alum used as adjuvant. For determining the right sensitization dose and the adequate number of challenges, we performed a literature research for existing protocols for ragweed sensitization. Based on relevant publications [73, 143, 165] we chose three different sensitization protocols and tested two different doses of ragweed pollen extract from commercial Allergon pollen. Additionally, to exclude effects of LPS, which is contained in the commercial pollen extract, we run a LPS control group.

4.3.1 Determination of allergen dose and challenge conditions

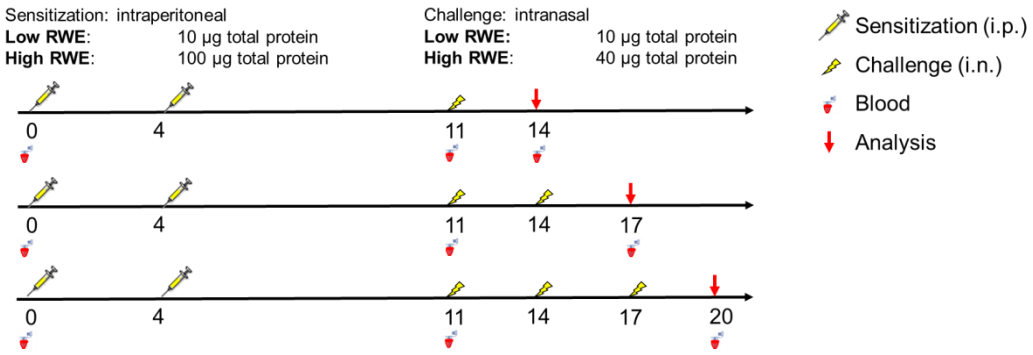

Figure 10: Experimental protocol for i.p. ragweed sensitization

Female, six weeks old BALB/c mice were sensitized by two i.p. injections of ragweed extract (RWE; commercial ragweed pollen extract from Allergon pollen [50mg/ml]) absorbed to 2 mg alum in a total volume of 200 µl, followed by one, two or three i.n. challenges with RWE with a volume of 20 µl (10 µl per nare). For sensitization, RWE was used in a high dose (100 µg total protein/mouse/injection) and in a low dose (10 µg total protein/mouse/per injection). For evaluation of effects of endotoxins, LPS was used in concentrations corresponding to the concentrations in the extract in the sensitization as well as in in the elicitation phase. Control mice were treated with PBS. Blood was taken on day 0, day 11 and before performing BAL.

4.3.1.1 Cell count in bronchoalveolar lavage (BAL) fluid

Three days after the last intranasal challenge, mice were sacrificed, the airways were lavaged and cells of the BAL fluid were analyzed for total and differential cell count of alveolar macrophages, lymphocytes, eosinophil and neutrophil granulocytes (Figure 11). Sensitization with a low dose as well as with a high dose of RWE led to an increase of the total cell number in BALF compared to the PBS control group (Figure 11A). Total cell number was higher with two or three intranasal challenges than with one challenge, for both, high dose and low dose sensitization. LPS in combination with alum had no effect on total cell number compared to PBS-treated animals.

The differential cell count shows that in non-ragweed treated animals (LPS controls, PBS control) more than 90% of total cells were macrophages (Figure 11B). In mice sensitized with a high dose of RWE an increase of eosinophils was detectable, which was even more obvious in low-dose sensitized animals (Figure 11D). Neutrophils showed a slight increase after low-dose sensitization and three intranasal challenges as well after treatment with a high dose of LPS (Figure 11).

Although there was a similar pattern in the composition of the inflammatory infiltrate of both, high dose and low dose sensitization, the low dose led to higher total cell counts and therefore shows a stronger phenotype of allergic asthma.

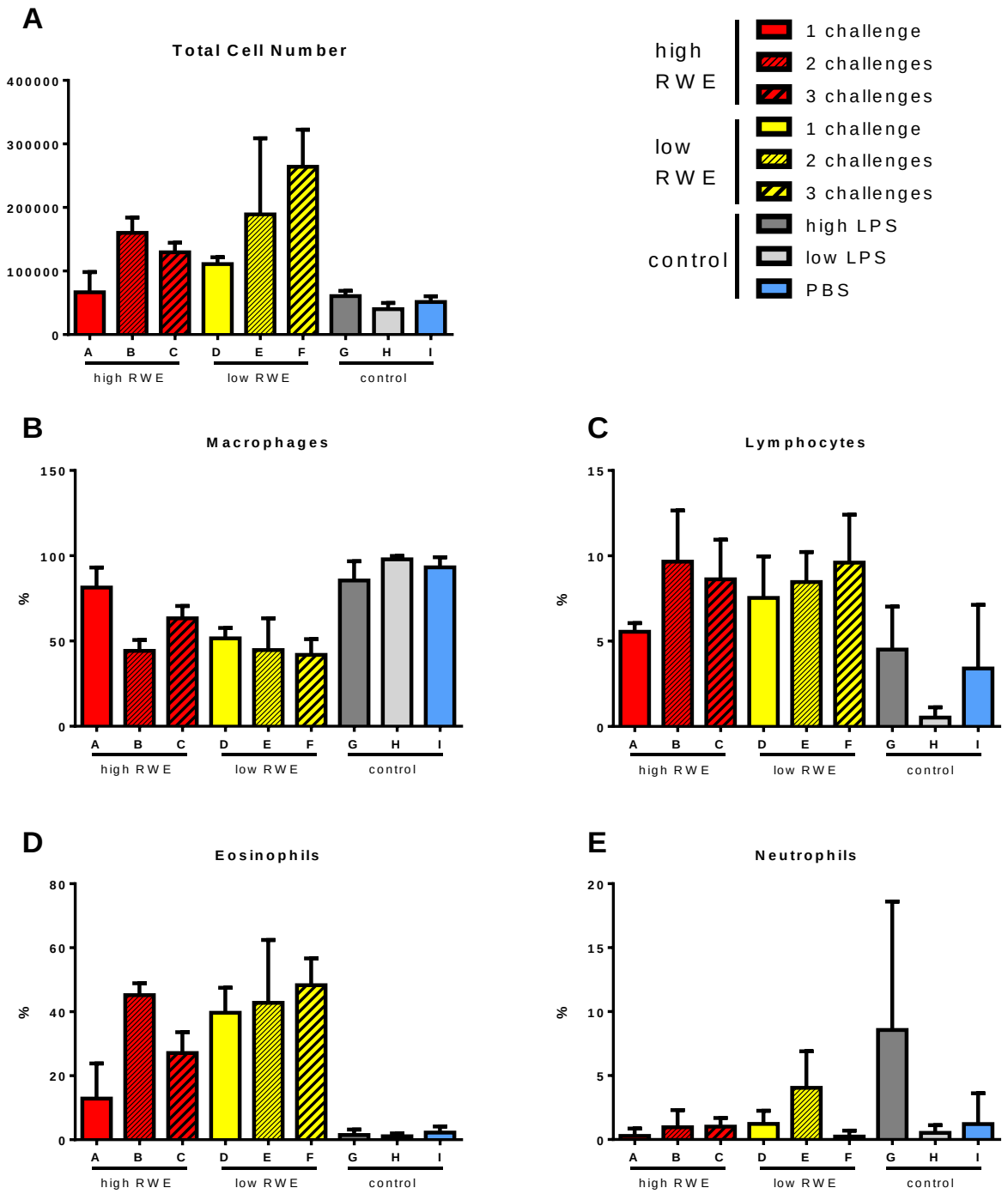

Figure 11: Total and differential cell count in BAL fluid

Female BALB/c mice were either sensitized with a high RWE dose (red bars) or with a low RWE dose (yellow bars) in combination with alum, control mice were treated with a high or low dose of endotoxin (LPS) or PBS in combination with alum. Afterwards mice were challenged intranasally 1, 2, or 3 times with a high or low dose of RWE or corresponding concentrations of LPS or PBS. 72 hours after the last challenge mice were evaluated for total and differential cell count in BAL fluid. Absolute numbers of total cell count (A) and the percentage distribution of differential cell count (B-E) were calculated. Results are expressed as mean ± SD (4 mice per group)

4.3.1.2 Lung histology

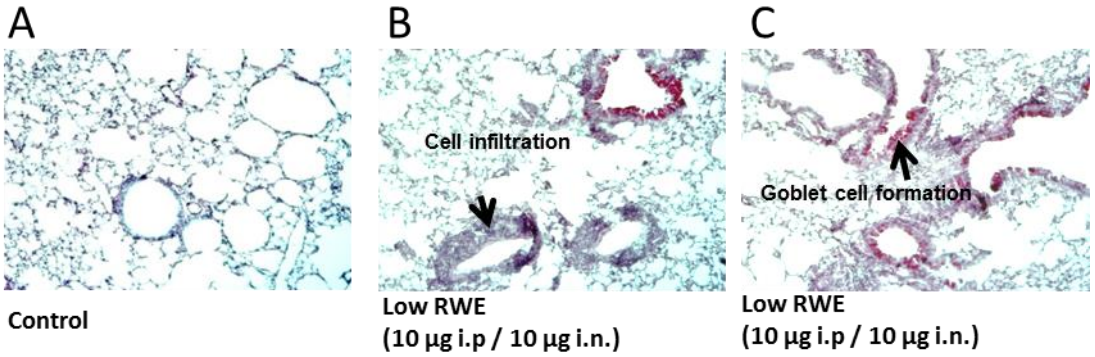

Figure 12: PAS staining of representative lung sections
Representative PAS stained lung sections of mice sham-treated with PBS (A) or sensitized with a low dose of RWE (B, C).

Low dose sensitization followed by intranasal administration of ragweed pollen extract induced allergic airway inflammation in lung tissue. Compared to mice, which were sham-treated with PBS/alum and intranasally instilled with PBS (Figure 12A), RWE-sensitized mice showed cell infiltration, goblet cell hyperplasia and mucus production in lung tissue (Figure 12B/C).

4.3.1.3 Immunoglobulin levels in plasma

Plasma samples were obtained at day 0, day 11 (before the first challenge) and 3 days after the last challenge and analyzed for immunoglobulins (Figure 13). In non-sensitized mice (LPS control groups, PBS control group) no major changes in immunoglobulin levels were observed. As shown in Figure 13A, low dose sensitization resulted in higher levels of total IgE than high dose sensitization, with levels increasing with each challenge. The same trend was seen for Amb a 1 spec. IgE (Figure 13B). Levels of Amb a 1 specific IgG_1 (Figure 13C) did not differ between low dose and high dose sensitization, but here was seen a clear increase with number of challenges. For Amb a 1 specific IgG_{2a} levels only high dose sensitization led to a clear increase (Figure 13D). Taken together, low dose sensitization leads to higher IgE levels, which indicate a Th2 sensitization, whereas high dose sensitization leads to higher levels of specific IgG_{2a}, suggesting a shift in Th1 direction.

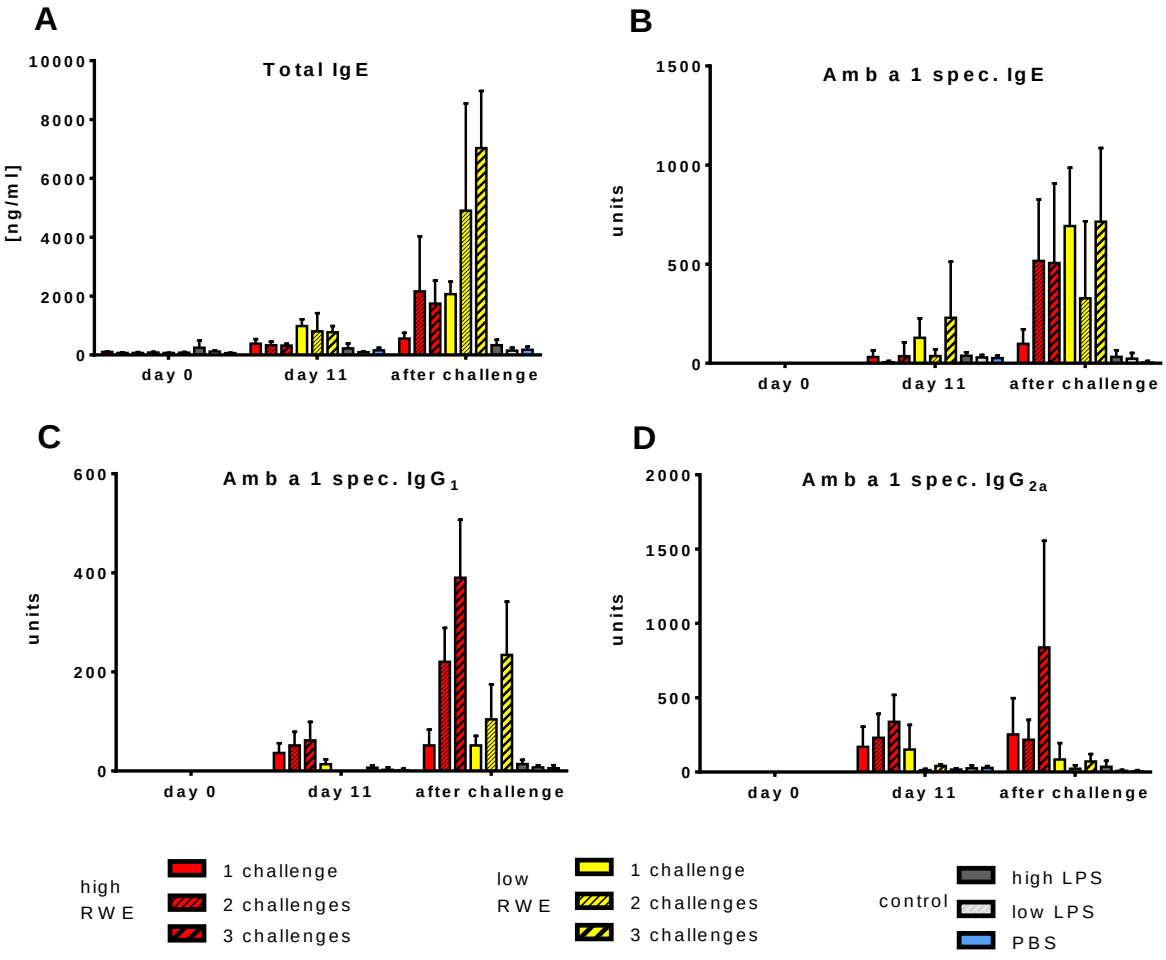

Figure 13: Immunoglobulin levels in plasma
Plasma was collected at indicated days (0, 11, and three days after the last challenge) and analyzed for total IgE (A), Amb a 1 specific IgE (B), Amb a 1 specific IgG_1 (C), and Amb a 1 specific IgG_{2a} (D). Data are expressed as mean ± SD (4 mice per group).

4.3.2 Prolongation of sensitization protocol

To improve the ragweed specific serology by reaching higher levels of immunoglobulins, we prolonged the established low-dose sensitization protocol. Mice were sensitized either according to the previous used low dose protocol (i.p. injections with 10 µg of RWE in combination with 2mg alum on day 0 and day 4 followed by three intranasal challenges on day 11, 14, and 17) or according to an extended protocol with four sensitizations on day 0, 7, 14, and 21 followed by three intranasal challenges on day 33, 36, and 39 (Figure: 14).

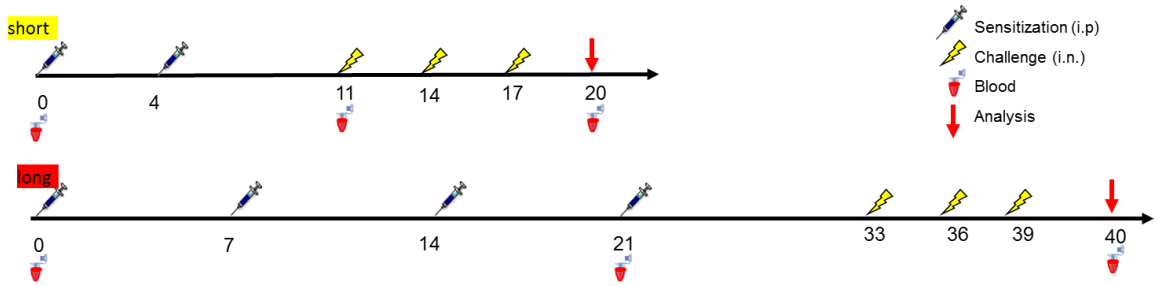

Figure: 14 Prolongation of sensitization protocol

Mice, sensitized according to the established protocol using a low dose of RWE (10 µg per mouse) and three challenges (short-term protocol) were compared to mice sensitized with a prolonged sensitization phase (four i.p infections once per week in combination with alum) followed by three intranasal challenges (long-term protocol).

BAL fluid was analyzed for total cell counts, macrophages, lymphocytes, eosinophils, and neutrophils (Figure 15A). Surprisingly, all cell counts, apart from neutrophils, were slightly higher after sensitization with the short term protocol. However, all cell numbers were at the same range and did not differ significantly between short term and long term sensitization. Additionally, only in cell-free supernatants of BAL fluids of long-term sensitized mice, the Th2 cytokines IL-4 and IL-5 were detectable (Figure 15B). The Th1 cytokine IFN-γ was detectable after long term sensitized mice as well, but was higher in control animals.

No immunoglobulins were detectable in sham-treated control animals. The levels of immunoglobulins in plasma of ragweed-treated mice were comparable between short term and long term sensitization (Figure 15C). For total IgE and RWE- and Amb a 1 specific IgE there was seen no difference, but for RWE- and Amb a 1-specific IgG_1 there was a trend for an increase after long term sensitization.

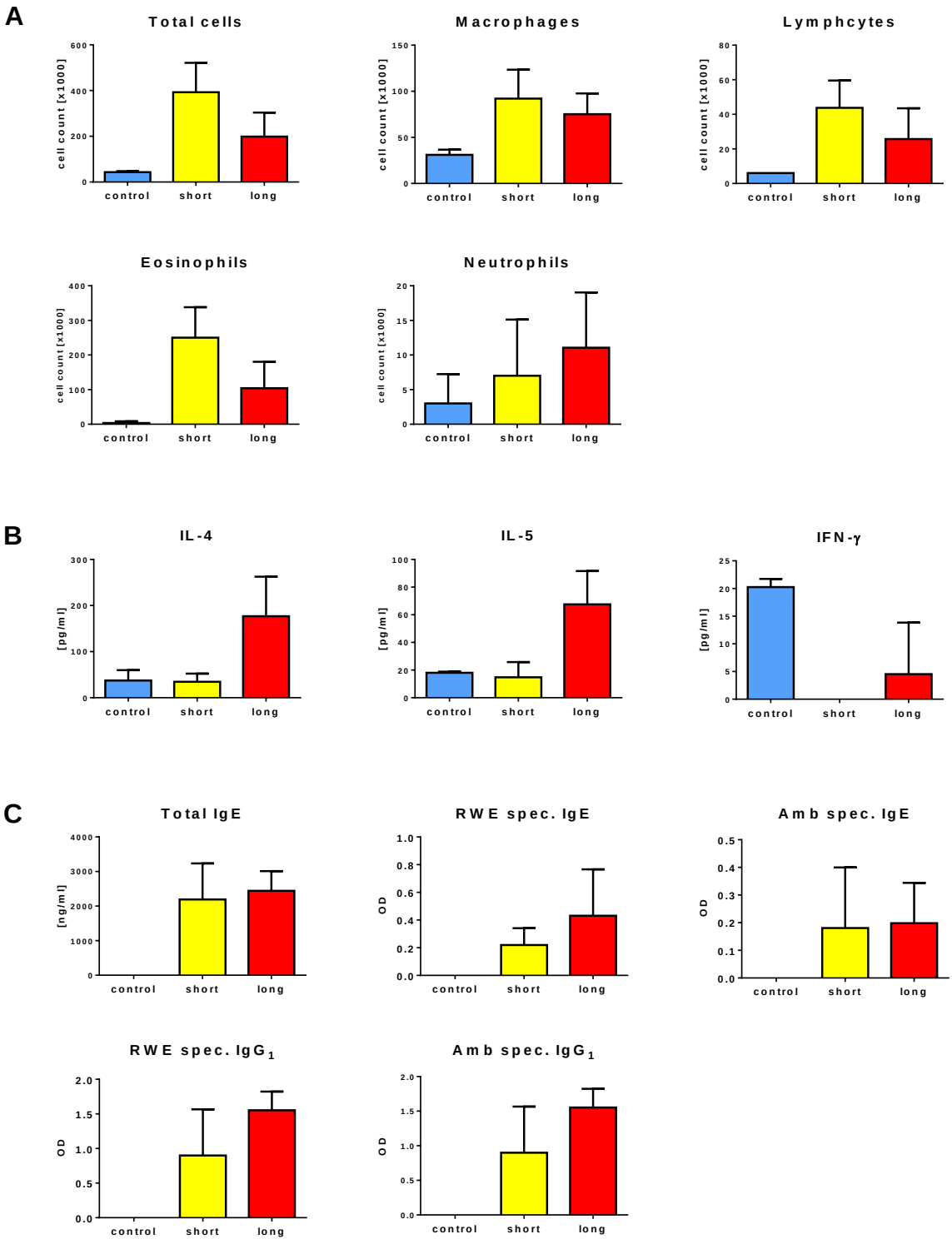

Figure 15: Comparison of cell count and cytokines in BAL fluid and immunoglobulins in plasma in short term and long term protocol

BALF of mice that were either sham-treated (blue bars), sensitized with the short term (yellow bars) or long term protocol (red bars) was analyzed for total and differential cell counts (A) and cytokines (B). Plasma of mice was analyzed for total IgE and specific IgE and IgG_1 (C). Data are represented as mean ± SD. n = 2 (control), 4 (short), 8 (long)

4.4 Analysis of the allergenicity of ragweed pollen grown under the influence of ozone

Ozone treatment of ragweed pollen was shown to increase pollen viability and ROS-generating NAD(P)H oxidases, which can enhance allergic sensitization and inflammation [98]. Therefore, we tested pollen grown under elevated ozone concentrations for their potential to induce symptoms of allergic airway inflammation and compared them to commercial available pollen or pollen grown under normal concentrations of ozone.

Commercial available pollen was obtained from Allergon (Sweden). Ozone treated pollen was grown at the Helmholtz Zentrum München as described in Methods section 3.1.1. Control pollen was grown under the influence of 40 ppb ozone, which is a normal ozone concentration in urban areas. Pollen grown under elevated ozone concentrations was treated with 80 ppb ozone, a concentration, which can be measured in inner cities on hot summer days frequently. The effects of that pollen on allergic airway inflammation were tested in the sensitization as well as the elicitation phase.

4.4.1 Sensitization phase

For evaluation of the effects of ozone treated pollen in the sensitization phase, pollen produced under three different growing conditions (commercial pollen, pollen grown at 40 ppb versus 80 ppb) were used. For the elicitation of allergic asthma by three intranasal instillations only pollen extract obtained from commercial Allergon pollen was applied. Mice were sensitized according to the established long term protocol (Figure 16). For sensitization three different doses of each pollen extract (low, medium, and high) were used (Table 15).

Figure 16: Sensitizing protocol
Mice were sensitized 4 times (day 0, 7, 14, 21) with different doses of either commercial Allergon pollen, pollen treated with 40 ppb ozone (control pollen), or pollen treated with 80 ppb ozone. On day 33, 36, and 39 mice were challenged intranasally with 20µl ragweed pollen extract of commercial Allergon pollen. Blood was taken on day 0, day 21, and day 40.

Group		µg protein/treatment	n [mice]
Control		-	8
Allergon	Low	15	6
	Medium	150	6
	High	1500	6
Ozone 40 ppb (ozone control)	Low	15	8
	Medium	150	8
	High	1500	8
Ozone 80 ppb	Low	15	8
	Medium	150	8
	High	1500	8

Table 15: Doses used for sensitization
Groups, concentrations, and numbers of mice, which were used for testing the effects of ozone-treated pollen on the sensitization phase.

As all experiments for the establishment of the model were run with commercial Allergon pollen, Allergon pollen was used as a positive control, to ensure that the experiments worked well. Allergon pollen is heavily loaded with lipopolysaccharides (LPS), which is not found on the freshly grown ozone treated pollen (Figure 6).

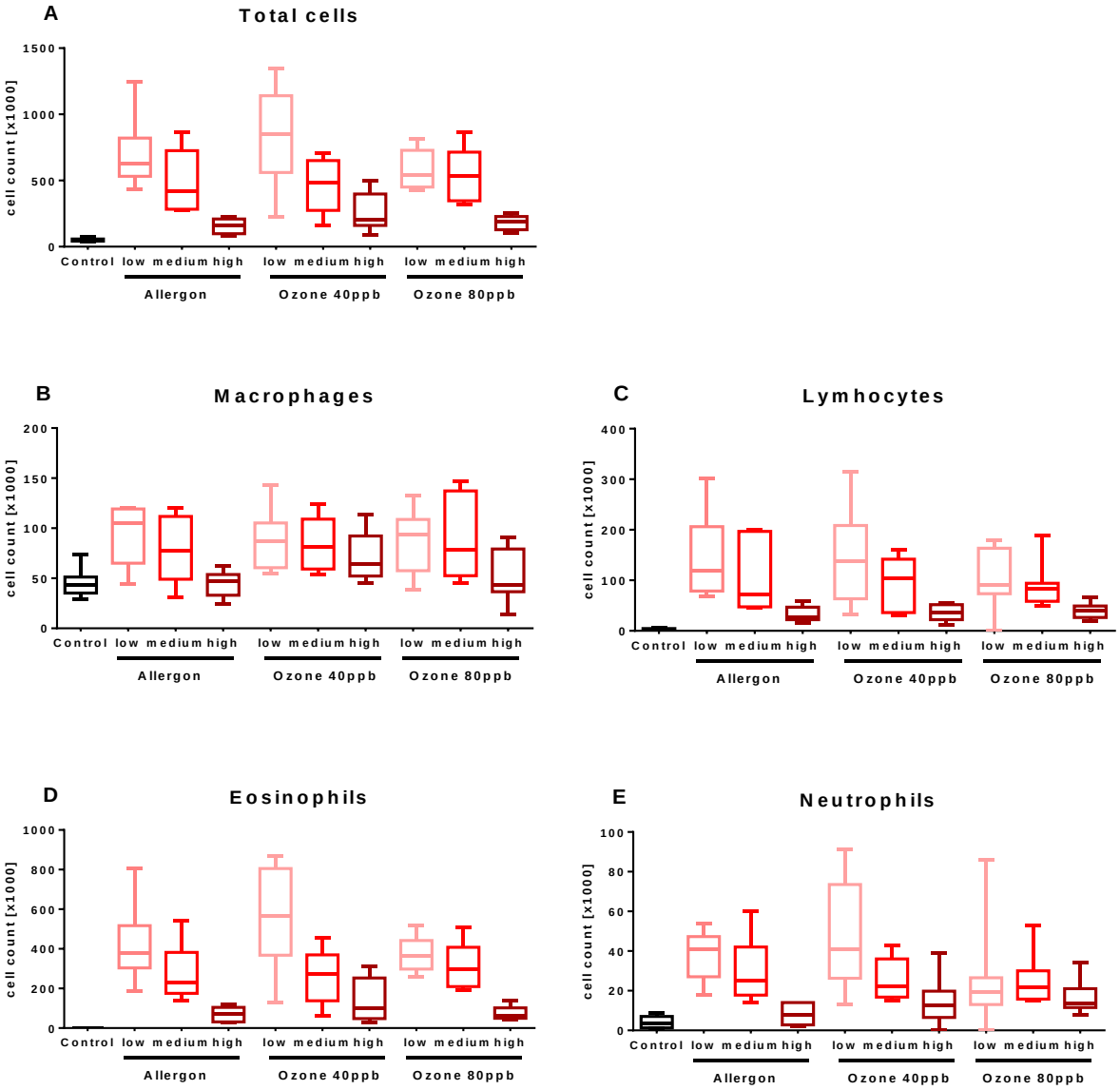

Figure 17: Cell infiltration in BAL fluid after sensitization with ragweed pollen grown under influence of different ozone concentrations
BALF of mice sensitized with Allergon pollen or with pollen treated with 40 ppb ozone or 80 ppb ozone respectively was analyzed for total cell count (A), macrophages (B), lymphocytes (C), eosinophils (D), and neutrophils (E). Data are represented as box boxplots. n = 6-8 mice per group

For all three pollen treatments the low dose sensitization (light red boxplots) resulted in the highest cell numbers, whereas high dose sensitization (dark red boxplots) led to a low absolute cell number (Figure 17A), suggesting that a too high dose in sensitization leads to development of tolerance. This effect was also seen on differential cell counts (Figure 17B-E). However, there was no statistical significant difference in absolute cell numbers or cell differential cell numbers between mice sensitized by commercial pollen, low ozone-treated pollen or high ozone-treated pollen. There was a slight tendency for a higher number of

eosinophils (Figure 17D) and neutrophils (Figure 17E) for mice sensitized with a low dose of 40 ppb ozone pollen compared to 80 ppb ozone pollen.

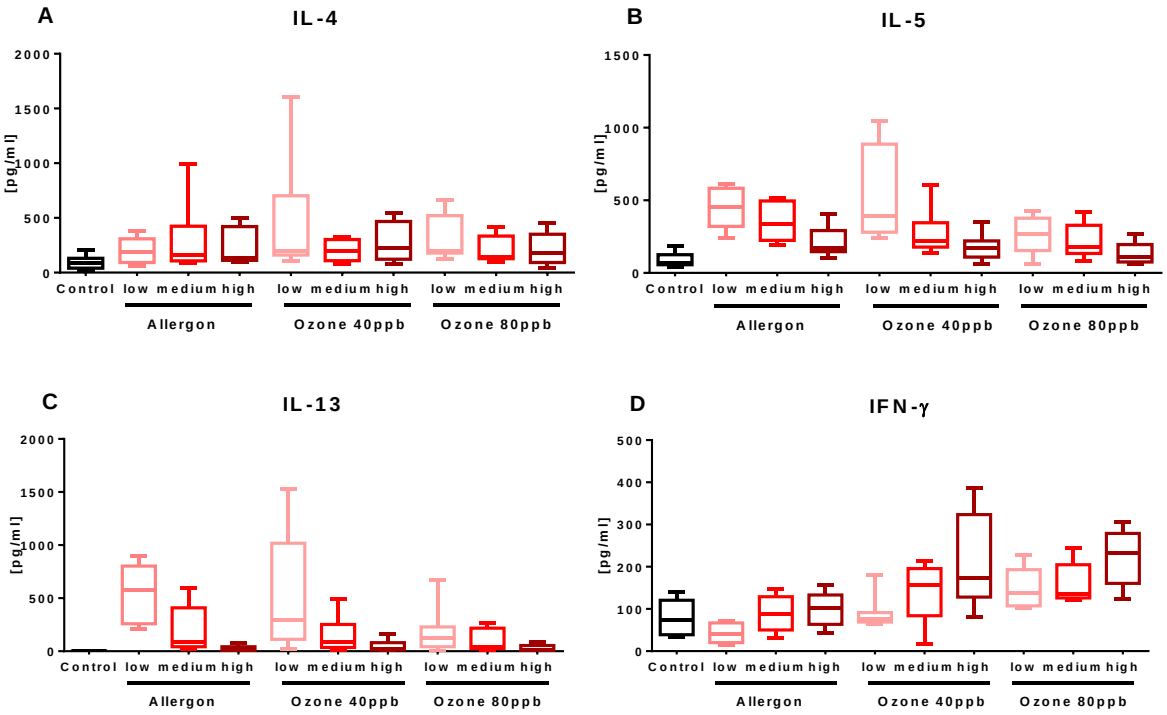

Figure 18: Cytokine levels in BAL fluid
The presence of IL-4 (A), IL-5 (B), IL-13 (C), INF-γ (D) in BAL fluids of mice sensitized with Allergon pollen, pollen treated with 40 ppb ozone, or pollen was analyzed by ELISA. Data are represented as box boxplots. n = 6-8 mice per group

The cytokine pattern in the BAL fluid was analyzed by ELISA. As shown in Figure 18A there was only a slight difference in IL-4 between non-sensitized control mice and sensitized mice. For IL-5 (Figure 18B/C) there was a higher cytokine level in BALF of mice sensitized with a low dose, a slight increase for mice sensitized with the medium dose and almost no difference between high-dose sensitized and non-sensitized mice. IFN-γ was produced in higher amounts after high dose sensitization (Figure 18D). Consistent with results from BAL cell count, also for cytokines there was the tendency for higher Th2 cytokines in BALF of animals sensitized with pollen grown under influence of 40 ppb ozone. In contrast, the Th1 cytokine IFN-γ tended to be higher in BALF of mice sensitized with pollen grown under the influence of 80 ppb ozone.

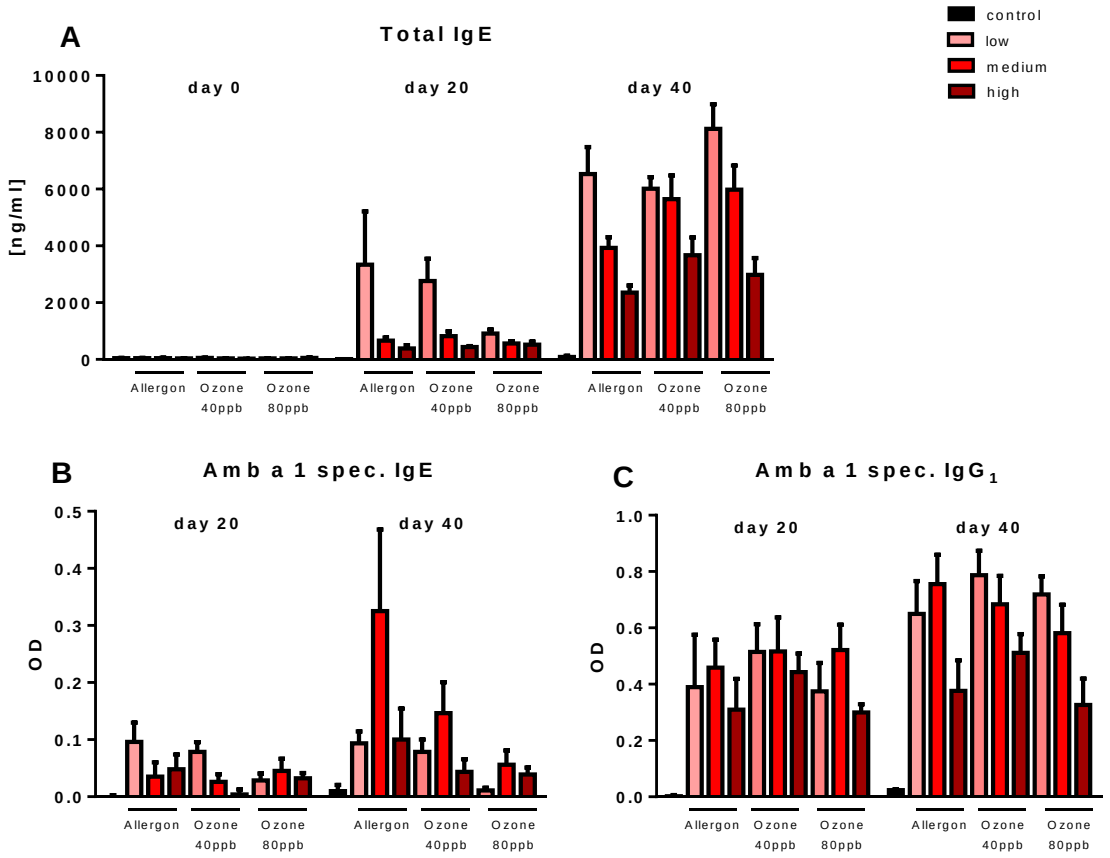

Figure 19 Plasma immunoglobulin levels
Plasma was analyzed for immunoglobulin levels by ELISA. On indicated days (0, 20, 40), blood samples were taken, plasma was obtained and total IgE (A), Amb a 1 specific IgE (B), and Amb a 1 specific IgG_1 (C) was determined. Data are expressed as mean + SEM; n = 6-8 mice per group

Plasma samples were taken before the sensitization started (day 0), after the sensitization phase (day 20) and after the last challenge (day 40) (Figure 19). Immunoglobulin levels of non-sensitized control mice were mostly under detection and showed no major changes. Sensitized mice showed an increase in total IgE and specific immunoglobulins. After the sensitization phase (day 20) low dose sensitization resulted in the highest levels of total IgE (Figure 19A). The same effect was observed on day 40, with low dose sensitization leading to the highest levels of total IgE and high dose sensitization resulting in the lowest levels of total IgE. No difference between the 40ppb and the 80 ppb ozone-treated pollen was observed. The same tendency but to a lower extent was seen for Amb a 1 specific IgE and Amb a 1 specific IgG_1 (Figure 19B/C) with higher levels in low-dose sensitization. Again, there were no differences in the effects of both ozone treatments.

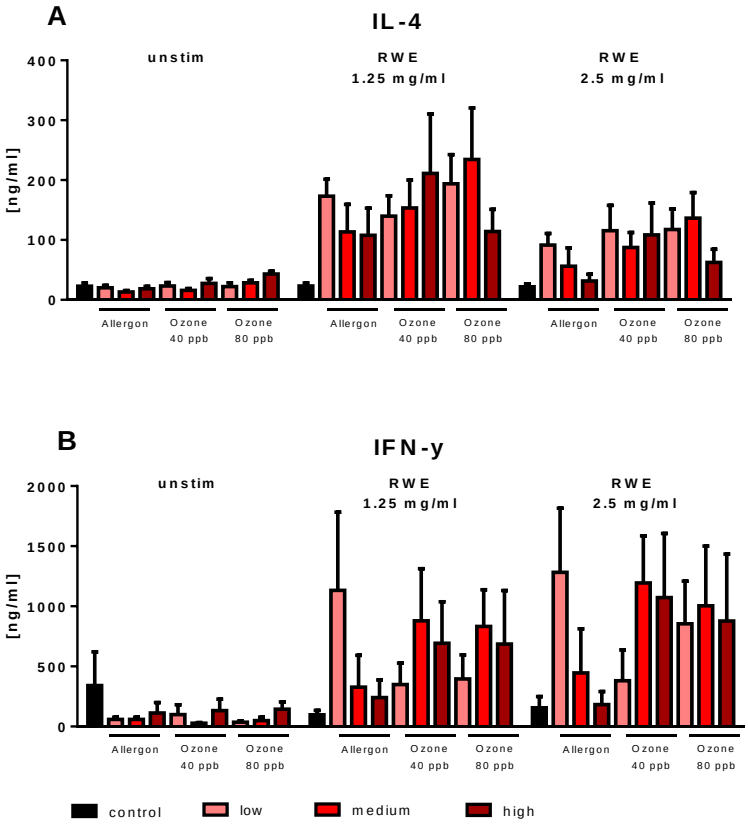

Figure 20: *Ex vivo* restimulation of splenocytes
Splenocytes of sensitized and non-sensitized mice were isolated and *ex vivo* restimulated with ragweed extract for 5 days. Supernatants were measured for IL-4 and IFN-γ. Data are expressed as mean + SEM; n = 6 mice per group

Furthermore, splenocytes of sensitized and non-sensitized mice were isolated and restimulated with two concentrations of ragweed extract (1.25 and 2.5 mg/ml). After five days of culture, supernatants were harvested and measured for IL-4 and IFN-γ (Figure 20). After restimulation with both ragweed doses there was secreted IL-4 as well as IFN-γ into the cell culture supernatant but there was no clear difference between splenocytes from mice sensitized with ozone-treated pollen or non-ozone-treated pollen.

4.4.2 Elicitation phase

As there was seen no difference between mice sensitized with pollen from plants grown under the influence of elevated ozone concentrations compared to mice sensitized with pollen grown under normal conditions in the sensitization phase, we next compared the influence of these pollen in the elicitation phase of allergic asthma.

Female BALB/c mice were sensitized according to the protocol used before (Figure 16). In this case, for the sensitizing phase commercial Allergon pollen was used for all groups in low dose (15 µg per mouse on day 0, day 7, day 14, and day 28) in combination with alum. Afterwards, for elicitation of allergic asthma the effect of Allergon pollen, pollen grown under normal ozone conditions (40 ppb ozone) and pollen grown under the influence of elevated ozone (80 ppb ozone) concentrations was compared. Therefore, mice were instilled on day 33, day 36, and day 39 with extracts of these different pollen and cell infiltration into BAL fluid and levels of immunoglobulins in plasma were evaluated.

All treatments elevated the total cell count in BAL fluid (Figure 21). The freshly grown pollen (ozone 40 ppb and ozone 80 ppb) exert a stronger effect on increasing total cells, macrophages, lymphocytes, eosinophils, and neutrophils as commercial available pollen (Allergon). However, no difference was seen between pollen grown under the influence of 40 ppb or 80 ppb ozone.

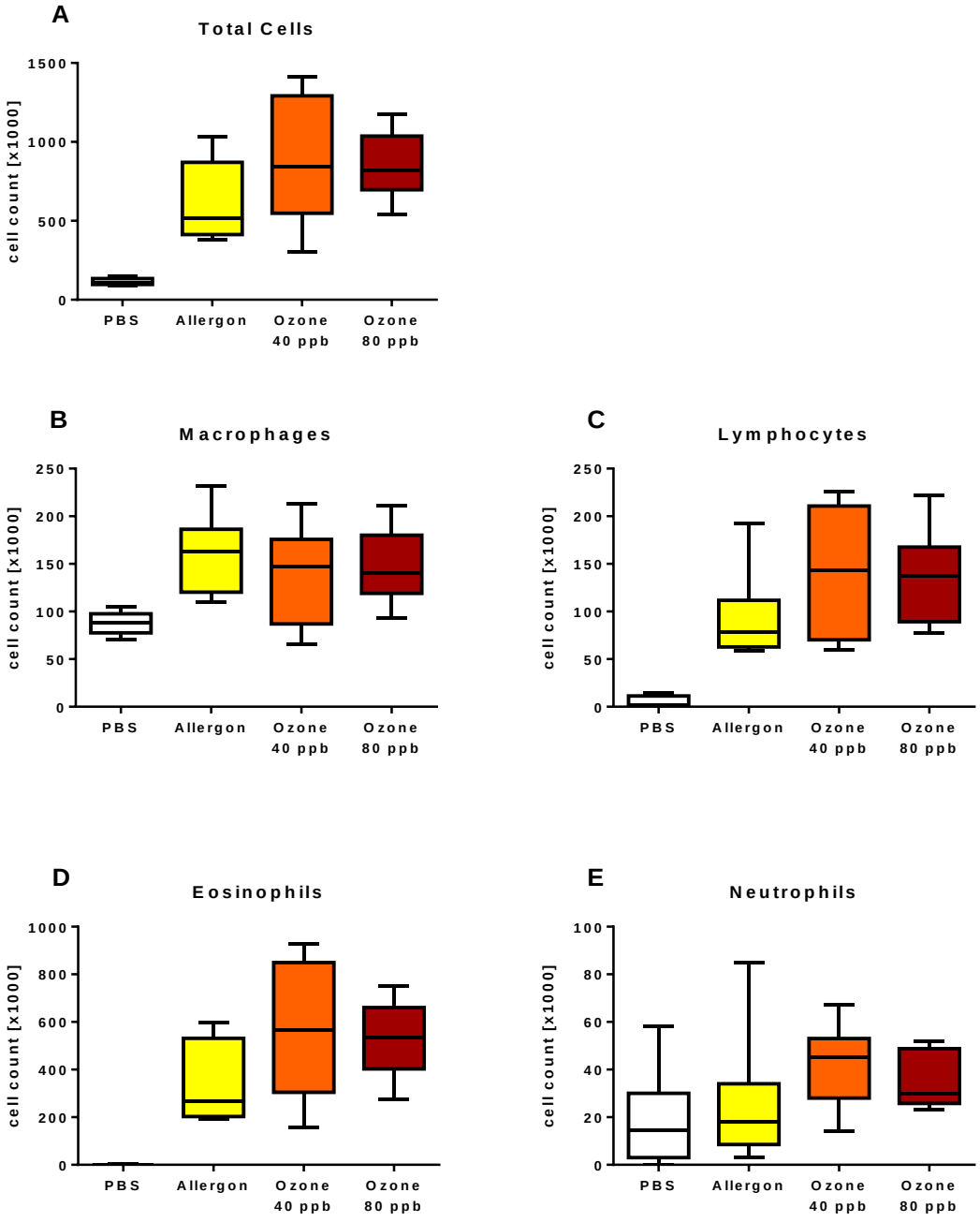

Figure 21: Cell infiltration in BAL fluid after challenge with ragweed pollen treated with different ozone concentrations in sensitized mice

BALF of sensitized mice challenged with Allergon pollen or with pollen treated with 40 ppb ozone or 80 ppb ozone respectively was analyzed for total cell count (A), macrophages (B), lymphocytes (C), eosinophils (D), and neutrophils (E). Data are represented as box boxplots. n = 8 mice per group

Plasma samples were obtained on day 0, day 20, and day 40 and analyzed for total IgE, Amb a 1 specific IgE and IgG_1 (Figure 22). For total IgE there was a slight tendency for higher levels after challenge with pollen grown under the influence of 40 ppb ozone compared to 80 ppb ozone (Figure 22A), which was more evident for Amb a 1 specific IgE (Figure 22B). For Amb a 1 specific IgG_1 there was no difference (Figure 22C).

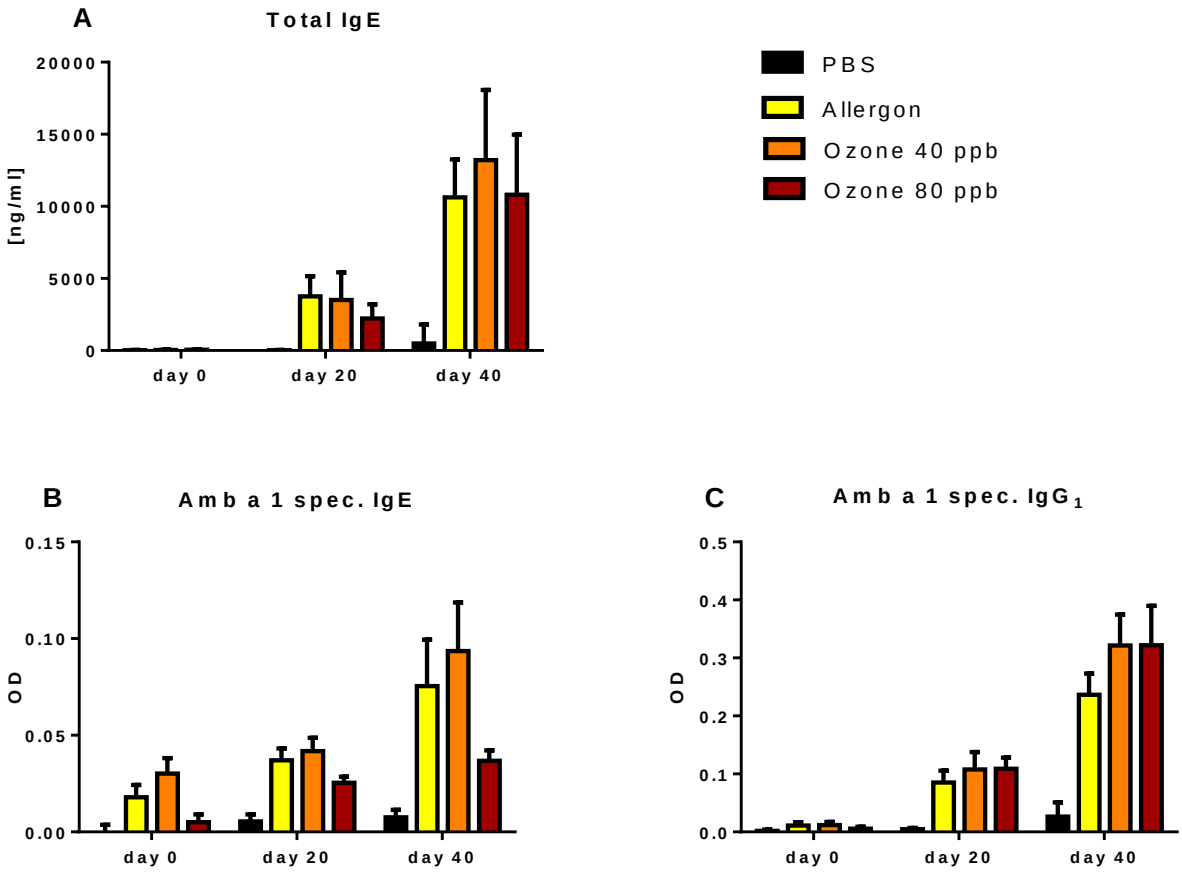

Figure 22: Plasma immunoglobulin levels
Plasma was analyzed for immunoglobulin levels by ELISA. On indicated days (0, 20, 40), blood samples were taken, plasma was obtained and total IgE (A), Amb a 1 specific IgE (B), and Amb a 1 specific IgG_1 (C) was determined. Data are expressed as mean + SEM; n = 8 mice per group

Taken together, systemic sensitization with ragweed extract in combination with alum followed by an intranasal challenge phase leads to a strong systemic Th2 sensitization as well as a local reaction in the lung. However, using this model no difference in sensitizing properties of pollen extracts form pollen grown under different ozone treatments was detectable.

4.5 Intranasal sensitization

Intraperitoneal injection of ragweed pollen extract in combination with alum as adjuvant followed by intranasal challenge leads to a strong Th2 response, with infiltration of cells into the lung and production of specific immunoglobulins. However, this represents not the physical way of exposure to pollen and does not mimic the natural route of sensitization. In order to imitate sensitization as it happens in nature, we established a protocol of intranasal sensitization, where mice are treated only intranasally with ragweed extract on 11 consecutive days.

4.5.1 **Establishment of intranasal sensitization**

For evaluation if a systemic Th2 response occurs in intranasally instilled mice, we compared a protocol of intranasal instillation to an i.p. sensitization protocol, from which is known to elicit a strong Th2 based response.

As shown in Figure 23, for the Th2 sensitization control mice were i.p. sensitized in combination with alum on day 0, day 7, and day 21 and treated 11 times with PBS intranasally (S/PBS). The intranasally sensitized groups were treated intranasally 11 times with ragweed extract (NS/RWE) or for control with PBS (NS/PBS).

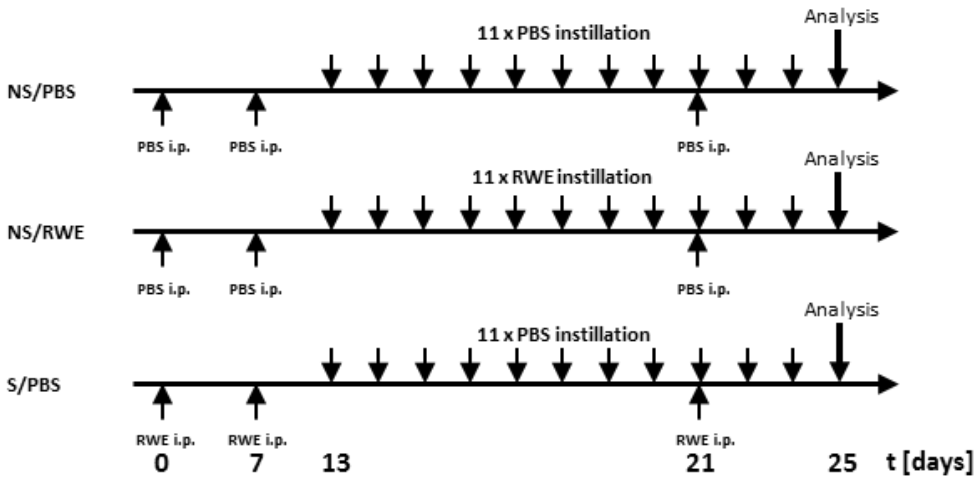

Figure 23: Comparison of sensitization by intranasal instillation and intraperitoneal injection

To show a ragweed specific Th2 response splenocytes were isolated and restimulated for 5 days, with OVA as control protein and with Amb a 1 and total ragweed extract for causing a ragweed specific reaction.

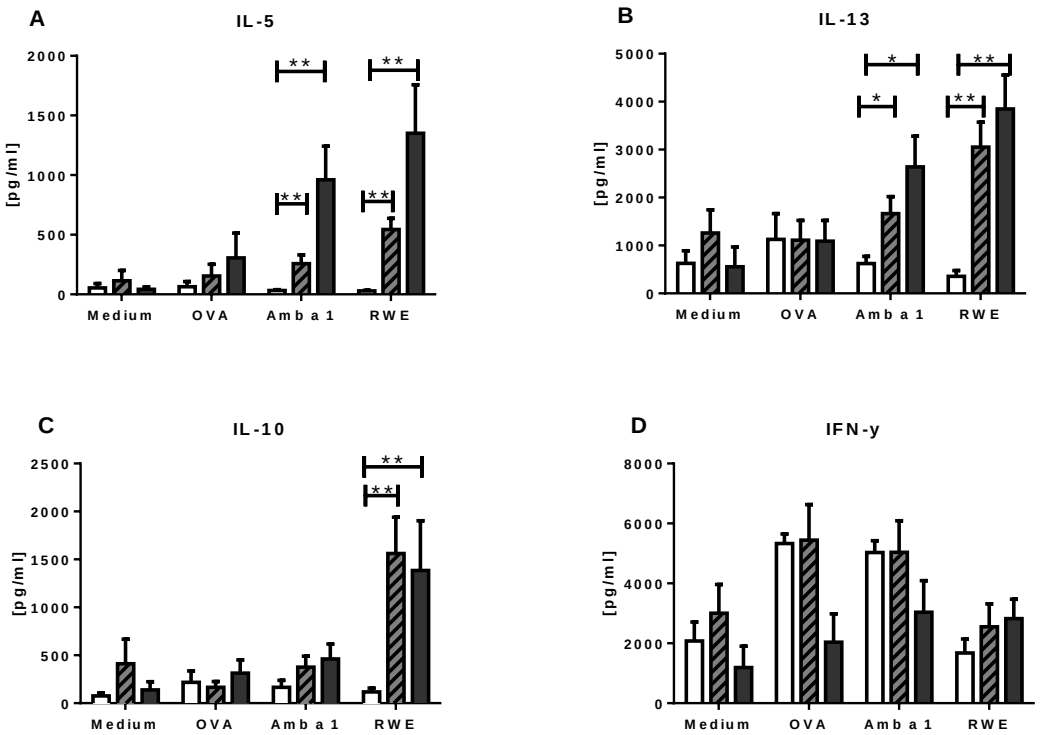

Figure 24: Eleven days of RWE instillation induce ragweed specific Th2-responses in splenocytes
Splenocytes were isolated and restimulated with medium, OVA, Amb a 1, or ragweed extract from non-sensitized PBS treated animals (white bars), non-sensitized RWE treated animals (shaded bars) and i.p. sensitized animals (black bars). After five days of culture IL-5, IL-13, IL-10 and IFN-γ were measured by ELISA. Data are represented as mean ±SEM with 6 mice per group. *$p \leq 0.05$, **$p \leq 0.01$ compared to PBS-controls.

As shown in Figure 24, after five days of restimulation, splenocytes of i.p. sensitized mice (black bars) secreted significantly increased levels of IL-5 and IL-13 after restimulation with Amb a 1 and significantly increased levels of IL-5, IL-13, and IL-10 after restimulation with ragweed extract compared to non-sensitized PBS instilled animals (white bars). However, this elevation of Th2 cytokines was not only seen for i.p. sensitized mice, but also for intranasally ragweed instilled mice. In contrast, the Th1 cytokine IFN-γ was elevated neither in i.p. sensitized nor in intranasally ragweed treated animals compared to control mice.

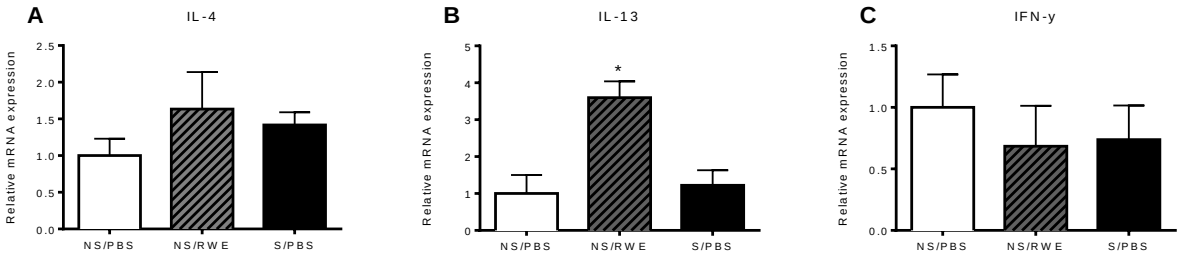

Figure 25: Eleven days of intranasal instillation of RWE induce Th2 micro milieu in lung tissue
Pulmonary tissue was analyzed for mRNA-expression of IL-4 (A), IL-13 (B), and IFN-γ (C). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$method. GAPDH: housekeeping gene. Data are expressed as mean + SEM. n=six mice per group. *p ≤ 0.05 compared to PBS-controls

In addition, we showed that the instillation of ragweed extract on 11 consecutive days led to an induction of a Th2 micro milieu in the lung tissue with slightly elevated levels of IL-4 mRNA (Figure 25A) and significantly elevated levels of IL-13 mRNA (Figure 25B) compared to control animals (white bars) or to only systemically i.p sensitized mice (black bars), which also showed no reaction in the lung. For gene expression of IFN-γ (Figure 25C) no differences were seen.

The selective increase of Th2 cytokine production in restimulated splenocytes showed that after 11 intranasal instillations of ragweed pollen extract a systematic Th2 sensitization is present as well as a local reaction in the lung tissue, confirmed by expression of Th2 cytokine mRNA.

Furthermore, cell infiltration into the lung was analyzed. In BAL fluid of intranasally treated animals total cell count was increased mainly due to elevated numbers of lymphocytes and eosinophils (Figure 26).

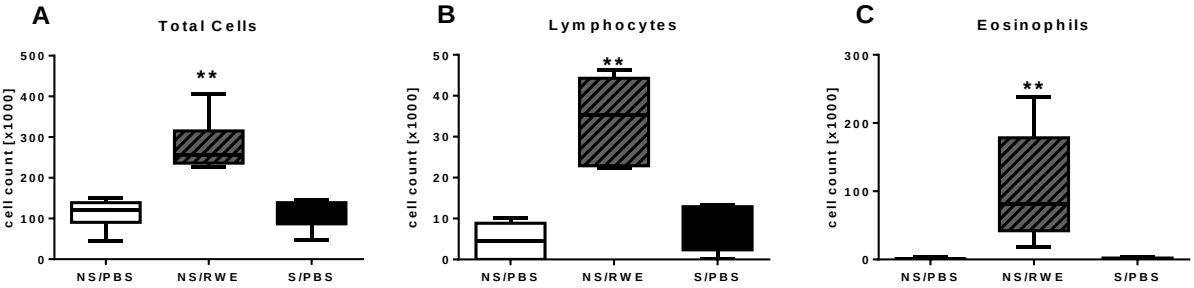

Figure 26: Eleven days of intranasal instillation lead to cell infiltration into the lung
BAL fluid of mice was analyzed for total cell count (A), lymphocytes (B), and eosinophils (C). Data are shown as boxplots. n=six mice per group. **p ≤ 0.01 compared to PBS-controls

To examine on which day the intranasal sensitization takes place, a time course was run and mice were instilled intranasally for one, three, eight, or eleven consecutive days with ragweed pollen extract.

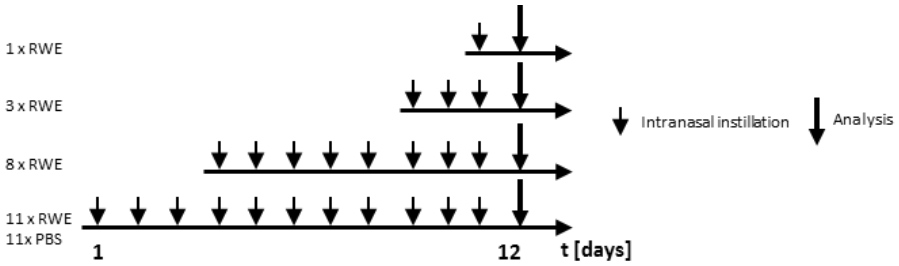

Figure 27: Evaluation of sensitizing properties of ragweed extract in a time course
Female, 10 weeks old BALB/c mice were treated intranasally with RWE either one, three, eight or eleven days. Control groups were treated eleven days with PBS or left untreated.

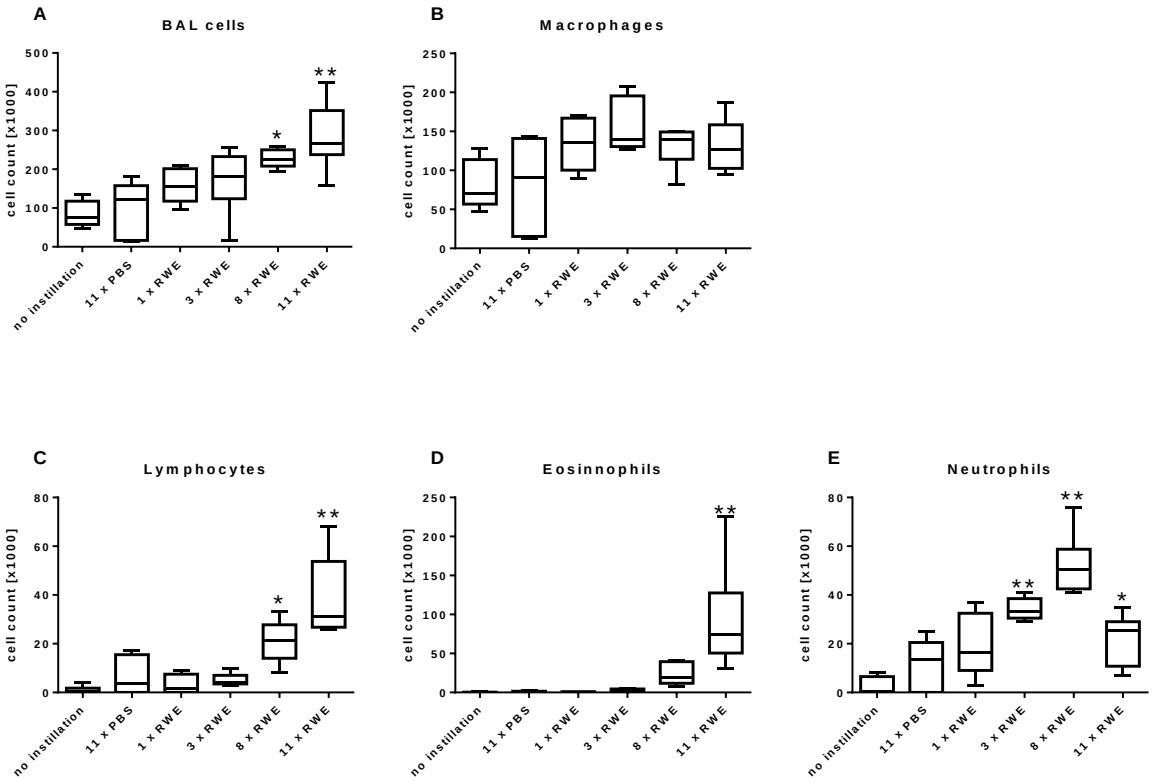

Figure 28: Cells of BAL fluid
BALF of mice that were instilled intranasally one, three, eight, or elven times was analyzed for total cell count (A), macrophages (B), lymphocytes (C), eosinophils (D), and neutrophils (E). Data are displayed as boxplots indicating minimum, 25% percentile, median, 75% percentile, and maximum. n=six mice per group. *p ≤ 0.05, **p ≤ 0.10 compared to untreated controls

The instillation time course showed that total cell number in BAL fluid increased with number of challenges (Figure 28A). Although number of macrophages did not differ much between all RWE-treated groups (Figure 28B), it was clear that lymphocytes and eosinophils (Figure 28C/D) reach the highest level on day 11, but neutrophils start to infiltrate on day 3 and reach the highest level on day 8 (Figure 28E). This early influx of neutrophils, followed by eosinophils is concurrent with literature [156], and shows that after eleven days of intranasal instillation there is a strong allergic reaction in the lung.

In lung tissue of control mice (Figure 29A/B) but also of mice instilled once with RWE (Figure 29C) there were no abnormalities. After three instillations (Figure 29D) cells started to infiltrate the lung tissue, after eight instillations (Figure 29E) slight mucus production by goblet cells is visible. After eleven intranasal ragweed instillations there was a strong cell infiltration and goblet cell hyperplasia (Figure 29F).

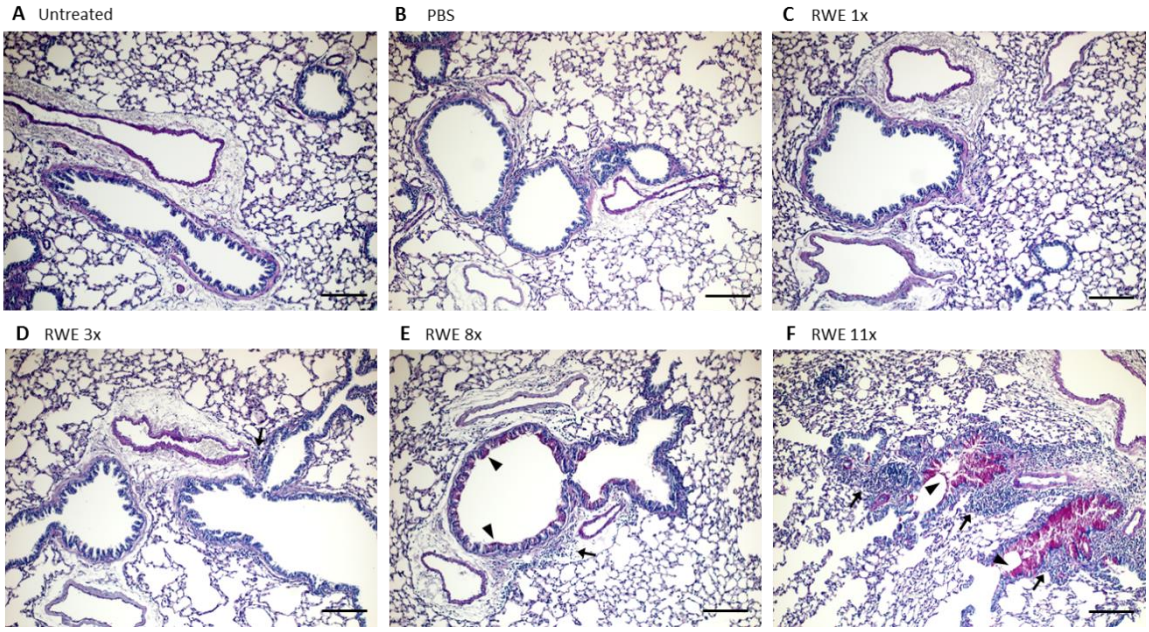

Figure 29: PAS staining of representative pulmonary sections of mice instilled according to the time course
Representative examples of pulmonary sections of PAS stained lungs. Arrows indicate inflammatory infiltrate, arrowheads mucus hyper secretion. Scale bar: 100 µm

Besides cell infiltration into BAL fluid and lung tissue, also gene expression in the pulmonary tissue was analyzed. There is a clear Th2 phenotype starting at day 8 and shown be high expression of IL-4 and IL-13 mRNA. There was no effect on the relative expression of mRNA of IFN-γ, GM-CFS, or TSLP.

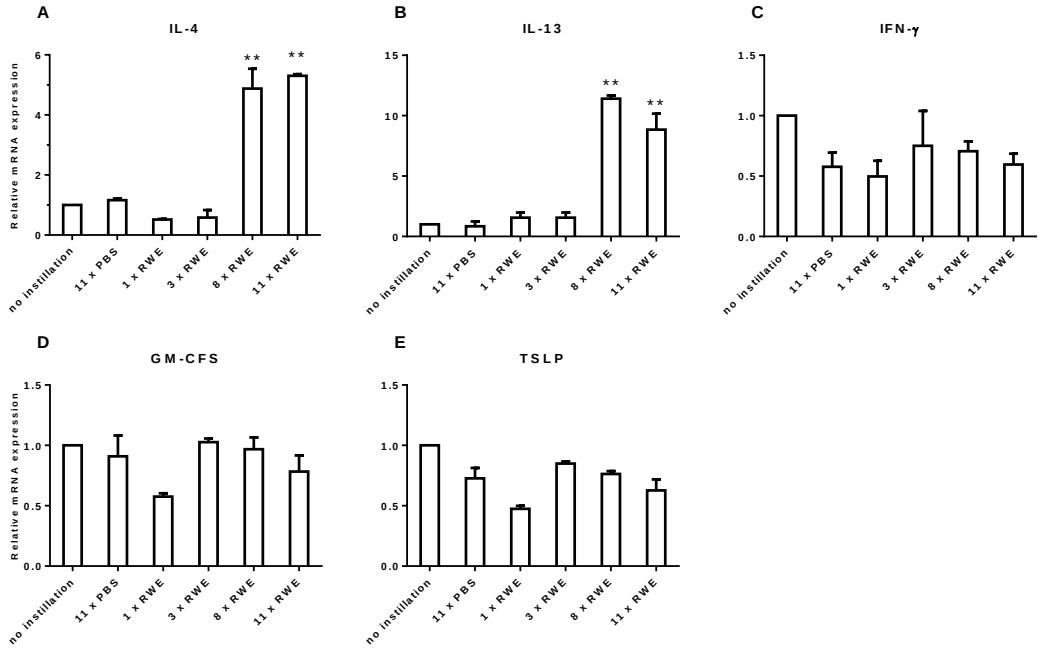

Figure 30: Quantitative real time PCR analysis of mRNA expression in pulmonary tissue
Pulmonary tissue was analyzed for mRNA-expression of IL-4 (A), IL-13 (B), IFN-γ (C), GM-CFS (D), and TSLP. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$method. GAPDH: housekeeping gene. Data are expressed as mean + SEM. n=six mice per group. **p ≤ 0.10 compared to untreated controls

On order to detect the production of immunoglobulins by B cells we extended the protocol with a three days intranasal challenge phase after a break of ten days and compared PBS-treated to RWE-treated mice.

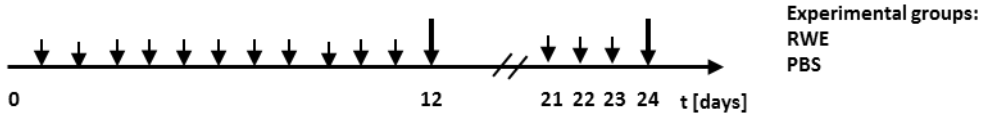

Figure 31: Extension of intranasal sensitization protocol
Mice were treated on eleven consecutive days with ragweed pollen extract or with PBS and after ten days break boosted with three intranasal treatments of ragweed extract or PBS, respectively. Blood was taken before the first treatment (day 0), on day 12 and one day after the last intranasal treatment (day 24.)

For total IgE there was a significant elevation on day 12 and a more obvious elevation on day 24 (Figure 32A). Although there was a slight elevation for total IgE in PBS-treated mice

(white bars), this trend did not reach statistical significance and could be due to the daily anesthesia. RWE-specific IgG_1 and Amb a 1 specific IgG_1 were not detectable in PBS-treated animals, but there was already an increase on day 12 and much stronger on day 21 in RWE-treated animals (Figure 32C/D).

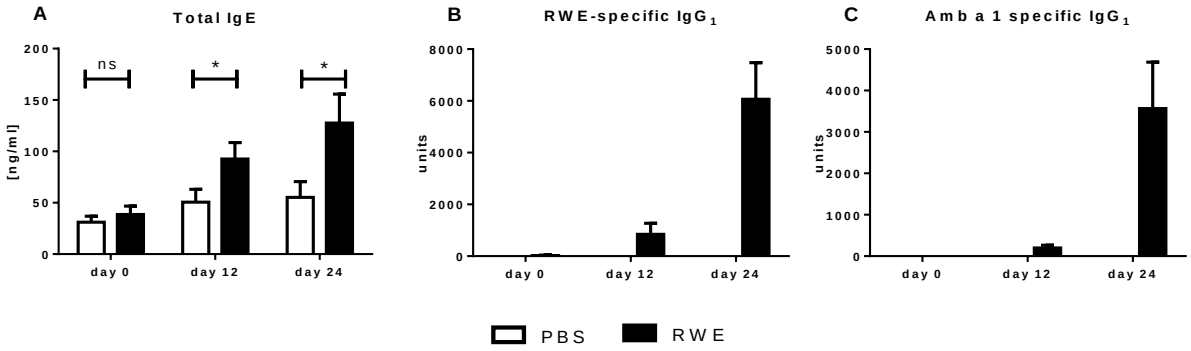

Figure 32: Immunoglobulin levels in plasma after elevation of instillation protocol
Blood was taken on day 0, day 12, and day 24 and plasma immunoglobulin levels were determined by ELISA. Data are represented as mean ±SEM. n = six mice per group; *p ≤ 0.05 compared to PBS group.

Altogether, eleven days of intranasal instillation led to a systemic sensitization, a local Th2 reaction in the lung and production of specific immunoglobulins in plasma.

4.5.2 Analysis of different compounds of the pollen extract

After showing the sensitizing potential of ragweed pollen extract systemically as well as locally, we were interested which compounds of ragweed pollen are responsible for its pronounced allergenicity.

4.5.2.1 Instillation total RWE, the major allergen Amb a 1 and the protein-free fraction

As described in Methods section 3.1.2 we gained a protein-free fraction (filtrate, all compounds <3kDa) from the total ragweed pollen extract by using a 3 kDa cut-off filter. As the molecular weights of all known ragweed allergens lies between 5 and 38 kDa [85, 166], no proteins and allergens should be located in the <3kDa fraction. To proof this, we tested the total ragweed extract, the filtrate, and the concentrate for the protein content by Coomassie

assay (see Methods sections 3.1.3). There was no difference between the protein content in total ragweed pollen extract and the >3kDa fraction, and there was no protein detectable in the <3kDa fraction (Figure 33).

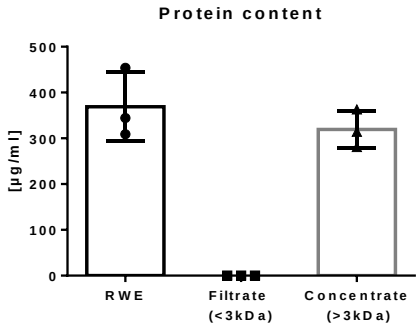

Figure 33: Measurement of protein content in total RWE, filtrate, and concentrate by Coomassie assay

To examine, which fractions of ragweed extract exert the sensitizing or proinflammatory effects, we treated female 10 weeks old BALB/c mice on eleven consecutive days with either PBS, Amb a 1 alone, the protein-free fraction, Amb a 1 in combination with the protein free <3kDa fraction (to mimic the composition of the total ragweed extract), and the total ragweed extract. Plasma samples were taken at day 0 and day 12 and mice we sacrificed 24 hours after the last instillation on day 12 (Figure 34).

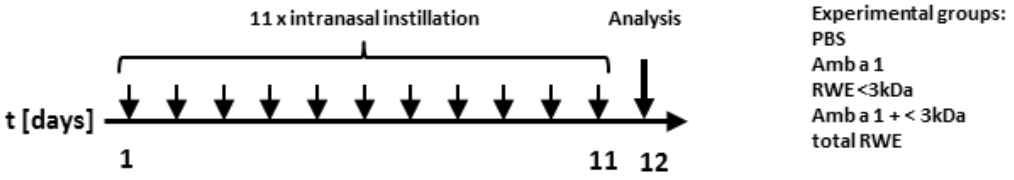

Figure 34: Experimental protocol for intranasal instillation of ragweed pollen extract
Mice were instilled on eleven consecutive days with either PBS, Amb a 1 alone, the protein free fraction alone, Amb a 1 in combination with the protein free <3kDa fraction, or the total ragweed pollen extract (RWE).

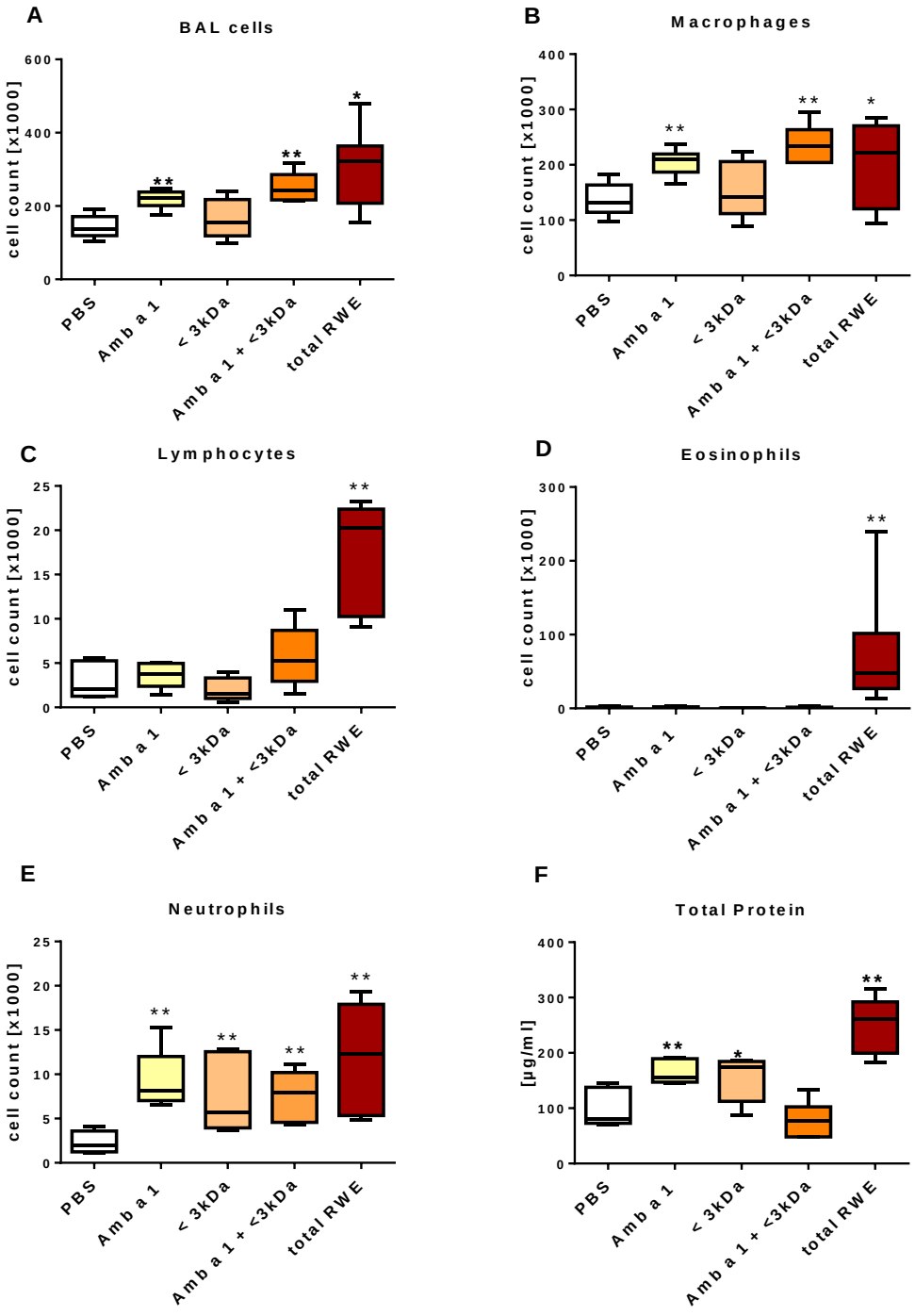

Figure 35: Intranasal instillation of ragweed extract fractions induces inflammatory BAL infiltrate
BALF of mice that were instilled 11 times intranasally was analyzed for total cell count (A), macrophages (B), lymphocytes (C), eosinophils (D), and neutrophils (E). Cell free supernatants of BALF were measured for total protein content by Coomassie assay (F). Data are displayed as boxplots indicating minimum, 25% percentile, median, 75% percentile, and maximum. n=six mice per group; *p ≤ 0.05, **p ≤ 0.01 compared to PBS-controls

Total BAL cell numbers were increased significantly as compared to controls (PBS) after instillation of Amb a 1 alone, Amb a 1 plus RWE <3 kDa, and total RWE. Instillation of the protein-free extract (RWE <3 kDa) alone did not increase total BAL cell numbers (Figure 35A). A similar result was obtained for the numbers of BAL macrophages (Figure 35B). Lymphocyte (Figure 35C) and eosinophil (Figure 35D) counts were only increased in animals treated with total RWE, whereas in animals treated with Amb a 1 or the protein-free fractions (RWE <3 kDa) no eosinophils and only very little lymphocytes were found. In contrast, BAL neutrophil numbers were increased significantly in all animals groups compared to PBS controls, including the group instilled with the protein-free extract (RWE <3 kDa) alone (Figure 35E). The protein content in the cell-free BAL fluid supernatant reached the highest level in mice treated with the total ragweed extract (Figure 35F).

Twenty-four hours after 11 consecutive intranasal instillations of total RWE, a strong inflammatory infiltrate and mucus hyper-secretion in the lungs was observed as compared to PBS controls (Figure 36E compared to A), whereas instillation of Amb a 1 alone, Amb a 1 plus the protein-free fraction (RWE <3 kDa) or the protein-free fraction alone, had no effects on lung histopathology (Figure 36B-D).

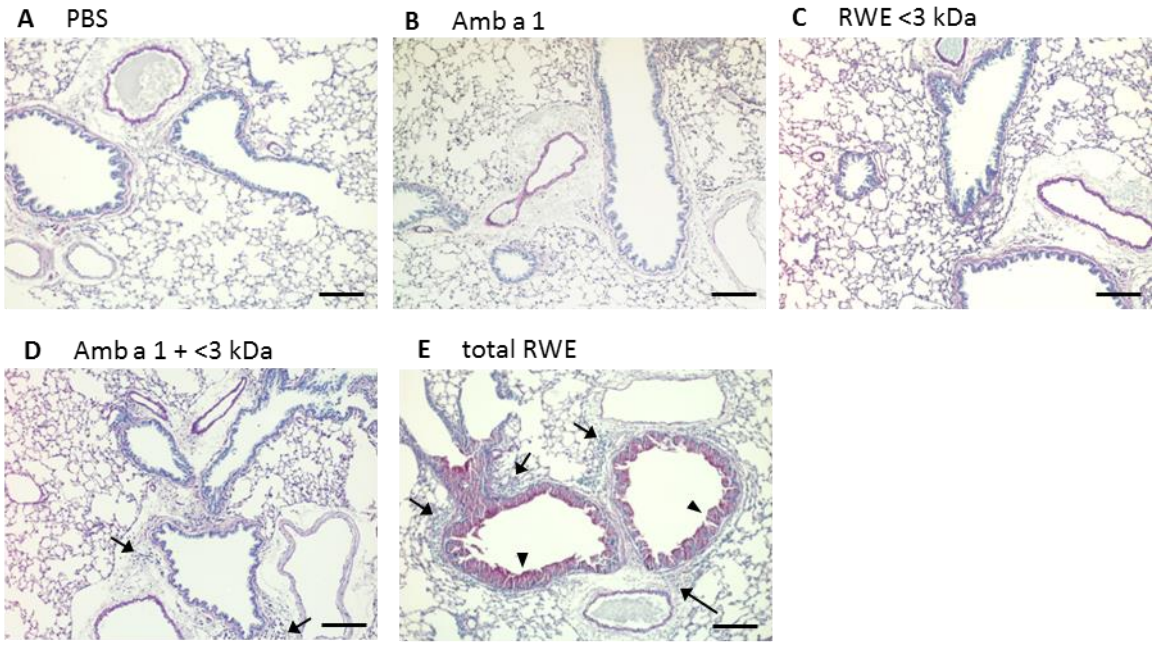

Figure 36: PAS staining of representative pulmonary sections
Representative pulmonary PAS-stained sections of mice, treated on eleven consecutive days with PBS (A), Amb a 1 (B), RWE <3kDa (C), Amb a 1 + <3kDa (D), or total RWE (E). Arrows indicate inflammatory infiltrate, arrowheads mucus hyper-secretion. Scale bar: 100 µm

To decipher the pulmonary micromilieu of intranasal sensitized mice, a quantitative real time PCR of the lung tissue was performed (Figure 37). Only the instillation of total RWE induced a significant increase of mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) and of IL-10, which can be secreted by Th2 cells, alternatively activated macrophages as well as by T_{reg} cells. On the contrary, no effect was shown on the expression of the Th1 cytokine IFN-γ or of the cytokine TNF-α. The marker for alternative activation of macrophages Arg1 was upregulated by total RWE, as well as mucins genes (Muc 2 and Muc 5ac). Furthermore, instillation of total RWE up regulated the expression of IL-22 and GM-CSF, but had no effect on IL-33 regulation. All investigated fractions of pollen extract had no significant effect on the regulation of the genes evaluated in this analysis.

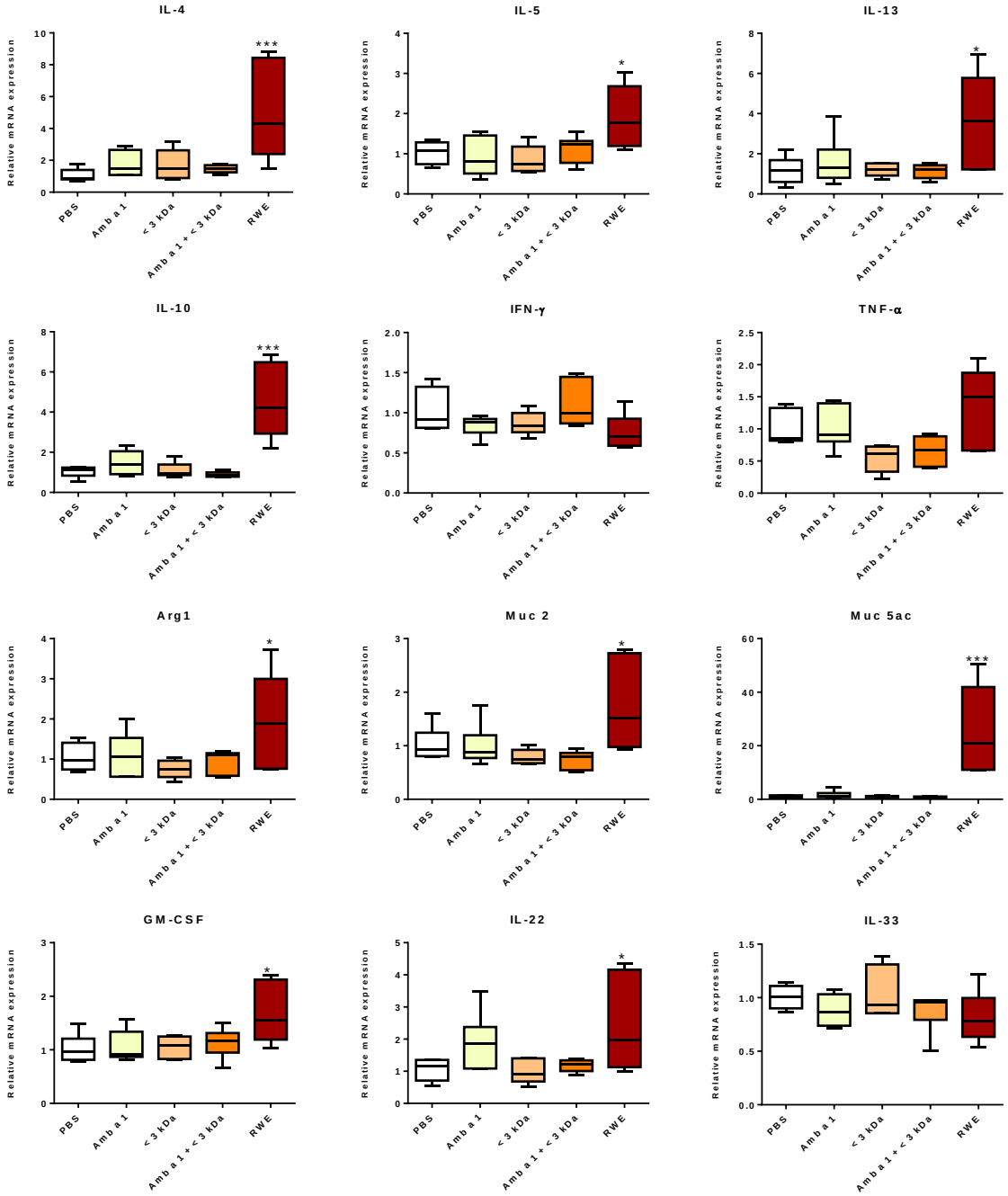

Figure 37: Quantitative real time PCR analysis of mRNA expression in pulmonary tissue
Pulmonary tissue was analyzed for mRNA-expression of indicated genes. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$method. GAPDH: housekeeping gene. Data are expressed as mean + SEM. n=six mice per group. *p ≤ 0.05 compared to PBS-controls.

Besides parameters of lung inflammation, we studied the induction of adaptive B cell responses by analyzing the effect of intranasal RWE-instillation on the levels of RWE-specific IgG_1 (Figure 38A) and Amb a 1-specific IgG_1 (Figure 38B) in plasma. A significant increase of

both, RWE specific-IgG_1 and Amb a 1 specific-IgG_1 was shown after instillation of total RWE, but not after instillation of the major allergen Amb a 1 alone. A slight increase of Amb a 1 specific IgG1 was observed after instillation of Amb a 1 in combination with the protein-free fraction.

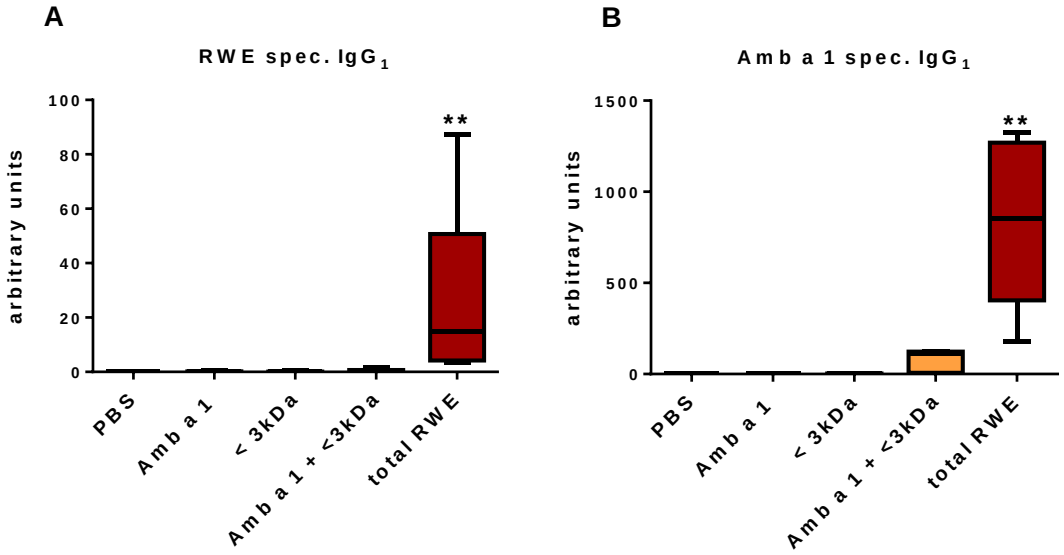

Figure 38: Specific IgG_1 levels in plasma after 11 days of intranasal instillation
Plasma levels of RWE-specific IgG_1 (A) and Amb a 1 specific IgG_1 (B) after eleven days of intranasal instillation. Data are represented as box boxplots. n = six mice per group; *p ≤ 0.05, **p ≤ 0.01 compared to PBS group.

4.5.2.2 Instillation of RWE depleted of adenosine

Adenosine was identified to be a potent immune regulatory substance in pollen [80]. As there were seen proinflammatory effects on the level of neutrophil infiltration not only by the total RWE, but also by the protein-free fraction (RWE <3 kDa), we analyzed if adenosine, a small compound contained in the aqueous pollen extract in high amounts, has an influence on intranasal sensitization. For the following experiments adenosine was used in the same concentrations as it is found in the RWE. The concentration of adenosine in the used RWE was determined by Ultra Performance Liquid Chromatography (UPLC) as 0.0327 mM. To remove adenosine from RWE, RWE was digested adenosine deaminase (ADA)

Mice were instilled either with PBS, the total RWE, RWE depleted of adenosine (RWE w/o ADO), or adenosine alone. After instillation with RWE depleted of adenosine total cells count in BALF was decreased significantly compared to mice instilled with the total extract (**Figure 39**A). This decrease was not due to the numbers of macrophages, because there was seen no difference between mice instilled with the total RWE or with RWE depleted of adenosine (Figure 39B). Although there were less neutrophils in BALF of mice treated with RWE depleted of adenosine compared to mice instilled with total RWE, this decrease did not reach statistical significance C). However, both, numbers of lymphocytes and of eosinophils, were reduced significantly by depletion of adenosine in the RWE (Figure 39D/E).

After instillation with RWE there is a strong increase of Amb a 1 spec. IgG_1 in plasma, as already described before. After 11 intranasal instillations with RWE depleted of adenosine, plasma levels of Amb a 1 spec. IgG_1 were significantly decreased compared to mice instilled with total RWE (Figure 39F).

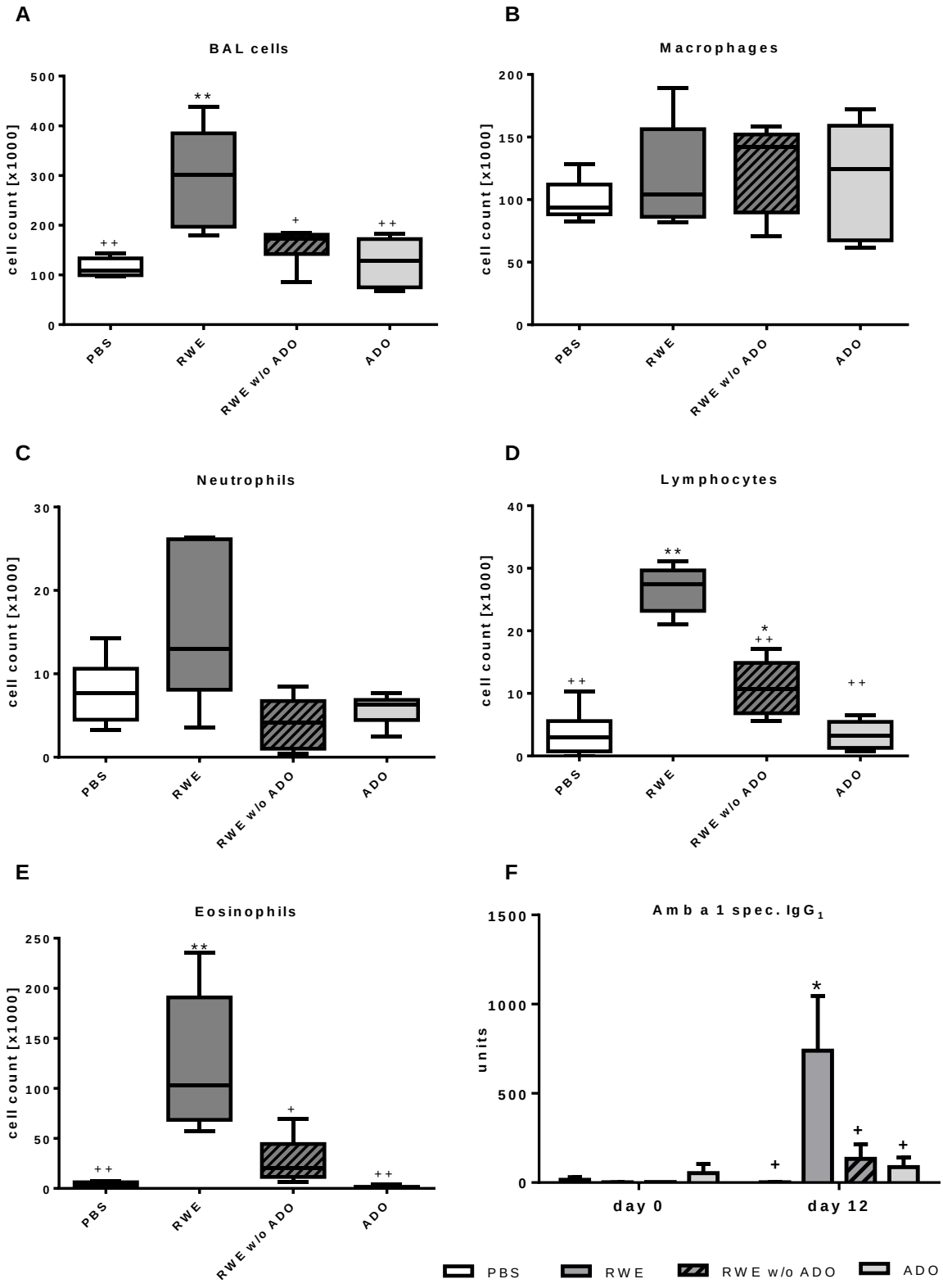

Figure 39: Depletion of adenosine in RWE decreases Th2 response in the lung after intranasal instillation
Mice were instilled with PBS (white bars), RWE (dark grey bars), RWE depleted of adenosine (shaded bars), or adenosine alone (light grey bars). BALF was analyzed for total cells (A), macrophages (B), neutrophils (E), lymphocytes (D), and eosinophils (E). Data are displayed as boxplots. n = six mice per group. F, In plasma Amb a 1 spec. IgG_1 was determined. Data are expressed as mean + SEM. n=six mice per group. ; *p ≤ 0.05, **p ≤ 0.01 compared to PBS group; +p ≤ 0.05, ++p ≤ 0.01 compared to RWE group

Splenocytes (Figure 40A) and cells of nose draining lymph nodes (Figure 40B) were isolated, *ex vivo* restimulated with medium, OVA (as control protein), Amb a 1, or RWE for five days. After restimulation with Amb a 1 there were elevated levels of IL-5 and IL-13 in supernatants of splenocytes and lymph nodes from mice instilled with total RWE, and even higher levels after restimulation with RWE. In supernatants of splenocytes and lymph nodes from mice instilled with RWE depleted of adenosine the production of IL-5 and IL-13 after restimulation with Amb a 1 or RWE was significantly decreased (grey shaded bars compared to dark grey bars). The Th1 cytokine IFN-γ was not increased after restimulation with Amb a 1 or RWE, but still there was a decrease of IFN-γ production for cells of mice treated with RWE depleted of adenosine.

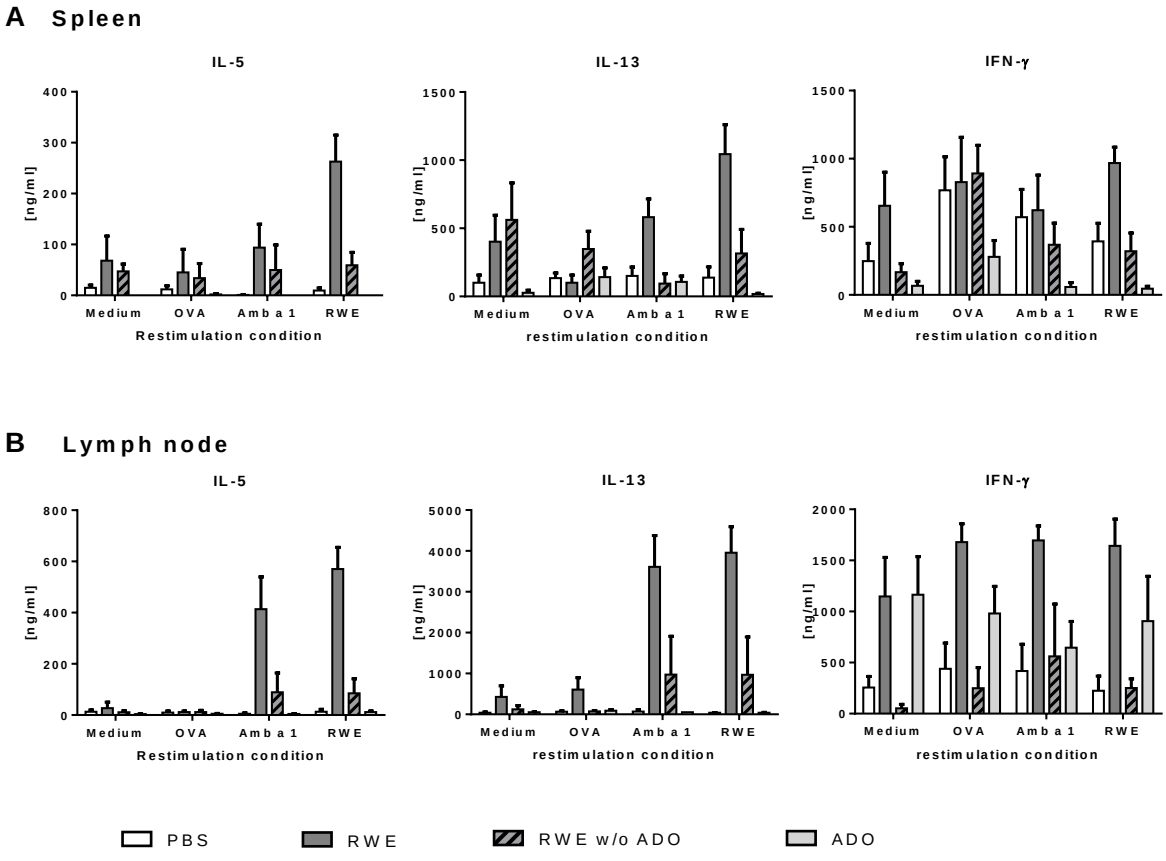

Figure 40: Depletion of adenosine leads to decreased production of Th2 cytokines after restimulation
Splenocytes (A) and cells of cervical lymph nodes (B) were restimulated with culture media, OVA [10 µg/ml], Amb a 1 [10 µg/ml] or RWE [1.25 µg/ml]. Five days later supernatants were analyzed for IL-5, IL-13, and IFN-y. Data are expressed as mean + SEM. n=six mice per group.

4.5.3 Bystander effect of intranasal sensitization

The proinflammatory and sensitizing effect of the ragweed pollen extract prompted us to analyze whether intranasally administered RWE might have an adjuvant effect on the elicitation phase of an unrelated, preexisting allergic immune response. To examine this, mice were OVA-sensitized (5 i.p. injections of OVA/alum). Before OVA-aerosol challenge, mice were instilled with RWE for eleven consecutive days. BAL was performed 24 hours and 7 days after the aerosol challenge, to cover an early as well as a late time point (Figure 41).

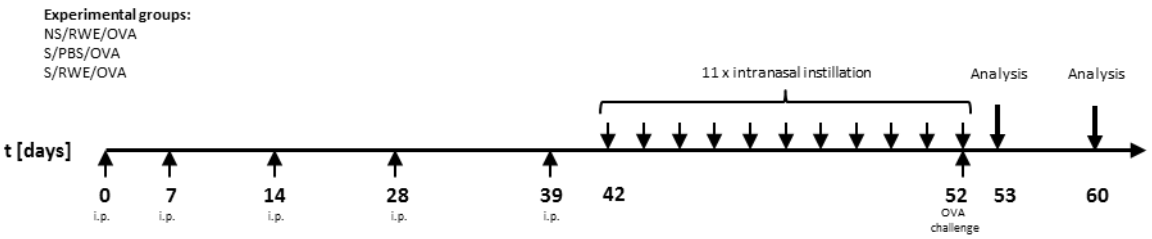

Figure 41: Analysis of bystander effect of intranasal instillation of RWE on preexisting OVA-sensitization
Mice were i.p. sensitized with OVA/alum (S) or control-treated with PBS/alum (NS), followed by intranasal treatment with ragweed pollen extract (RWE) or PBS. Finally, mice received an OVA-aerosol challenge.

To evaluate the bystander effect of intranasal instillation of ragweed extract on a preexisting OVA-sensitization, for elicitation of OVA-specific allergic airway inflammation only one OVA-aerosol challenge was performed, to achieve a very mild OVA-specific reaction.

In these OVA-sensitized mice, RWE instillation before OVA-aerosol challenge caused a significant enhancement in BAL inflammatory cells compared to mice, which were treated intranasally with PBS, one day (white bars) as well as seven days (black bars) after the OVA-aerosol challenge (Figure 42A). Moreover, the aggravated response in lungs of RWE-instilled mice was characterized by significantly elevated numbers of macrophages (Figure 42B) and lymphocytes (Figure 42D) one day after OVA-aerosol challenge, but not neutrophils (Figure 42C). The most dramatic increase was observed for eosinophils, which were already significantly elevated one day after OVA-challenge, but reached excessive numbers seven days after OVA-aerosol challenge in intranasally RWE-treated mice (Figure 42E).

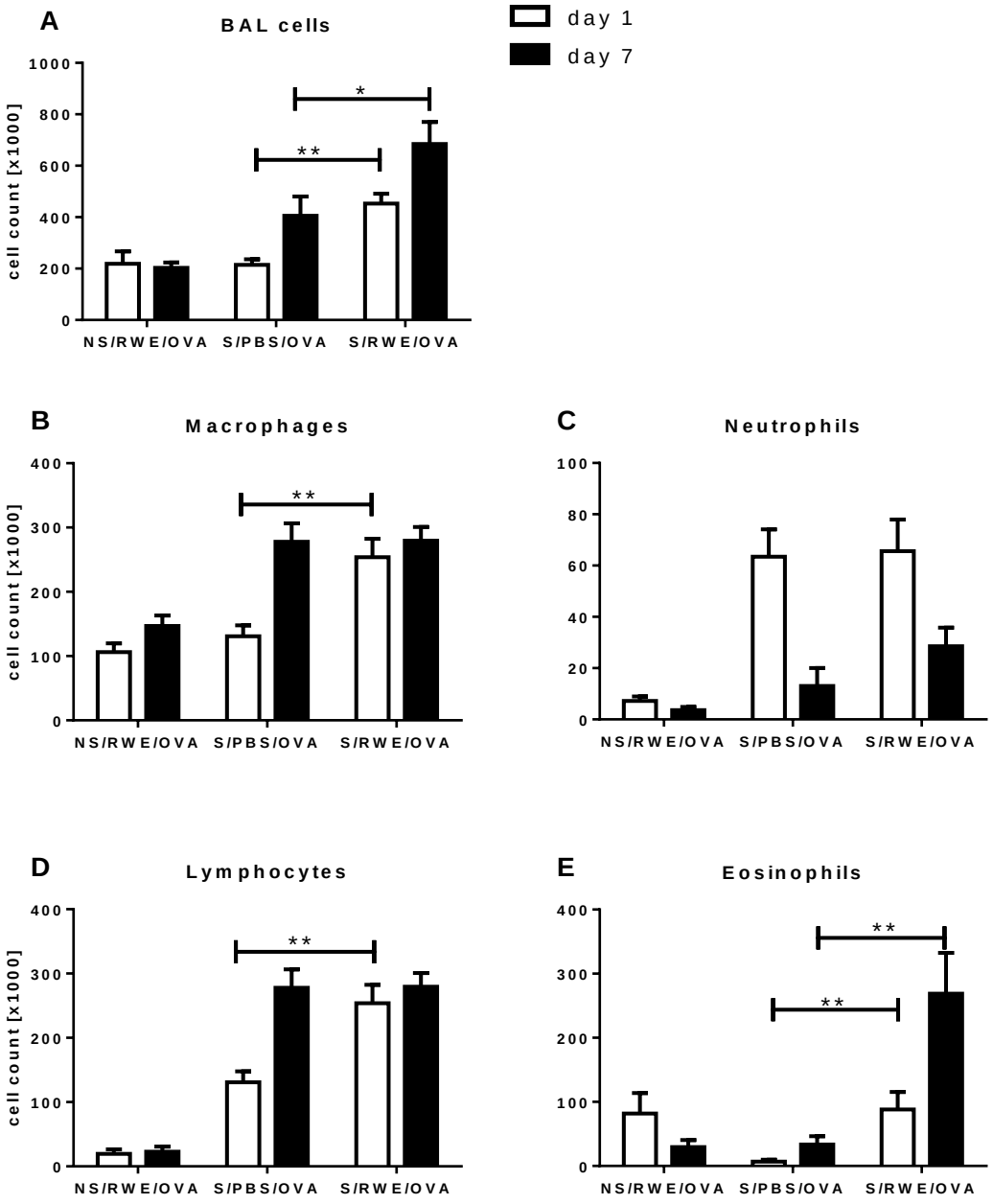

Figure 42: Intranasal instillation of RWE aggravates OVA-specific lung inflammation in OVA-sensitized mice

BALF was analyzed for total cell count (A), macrophages (B), neutrophils (C), lymphocytes (D), and eosinophils (E) one day (white bars) and seven days (black bars) after the OVA-aerosol challenge. Data are displayed as boxplots. n=six mice per group. *p ≤ 0.05, **p ≤ 0.01 compared to PBS-controls.

In immunohistology, one day after the OVA-aerosol challenge cells infiltrated in lungs of mice, which were non-OVA sensitized and RWE-instilled (NS/RWE/OVA; Figure 43A) as well as in mice, which were OVA-sensitized (S/PBS/OVA, Figure 43B). In lung tissue of mice, which

were both, OVA-sensitized and RWE-instilled (S/RWE/OVA) a much more pronounced lung inflammation with strong cell infiltration and goblet cell hyperplasia was observed (Figure 43C). Seven days after the OVA-aerosol challenge in RWE-treated mice no signs of inflammation were visible anymore. (NS/RWE/OVA, Figure 43D). In lungs of previously OVA/alum i.p.-sensitized mice seven days after the OVA-aerosol challenge, only a mild inflammatory infiltrate and mucus production was present (S/PBS/OVA, Figure 43E). In contrast, in previously OVA/alum i.p.-sensitized mice, which were additional intranasal RWE-instilled a single OVA-challenge led to exacerbated peribronchial and perivascular inflammation and mucus production (S/RWE/OVA, Figure 43F). This finding indicates an adjuvant activity of RWE on the extent of an unrelated bystander inflammation.

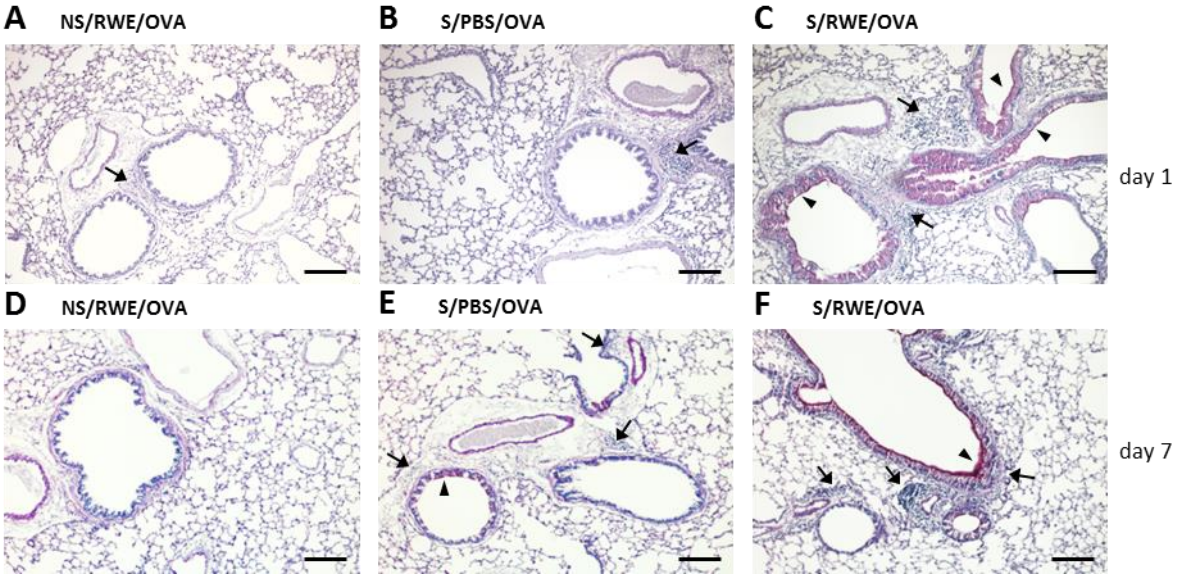

Figure 43: PAS staining of representative pulmonary sections
Representative pulmonary sections of PAS stained lung sections of mice, one day and 7 days after the OVA-aerosol challenge. Arrows indicate inflammatory infiltrate, arrowheads mucus hyper-secretion. Scale bar: 100 µm

Further we examined, if ragweed pollen extract has an increasing effect on the levels of total IgE and OVA-specific IgE in plasma. In non-sensitized mice, instillation of RWE followed by OVA-aerosol challenge had no influence on levels of total IgE (NS/RWE/OVA; Figure 44A). In OVA-sensitized mice, intranasal instillation of RWE followed by OVA-aerosol challenge (S/RWE/OVA) resulted in elevated levels of total IgE in plasma compared to mice instilled intranasally with PBS (S/PBS/OVA). This tendency was seen one day (Figure 44A, white bars) as well as seven days after the aerosol challenge (Figure 44A; black bars). These results indicate that intranasal instillation of RWE has an adjuvant effect on secretion of IgE in

sensitized individuals. For OVA-specific IgE (Figure 44B) there was seen a slight increase on day one for RWE-treated mice (white bars), but not on day seven (black bars) compared to PBS-treated mice.

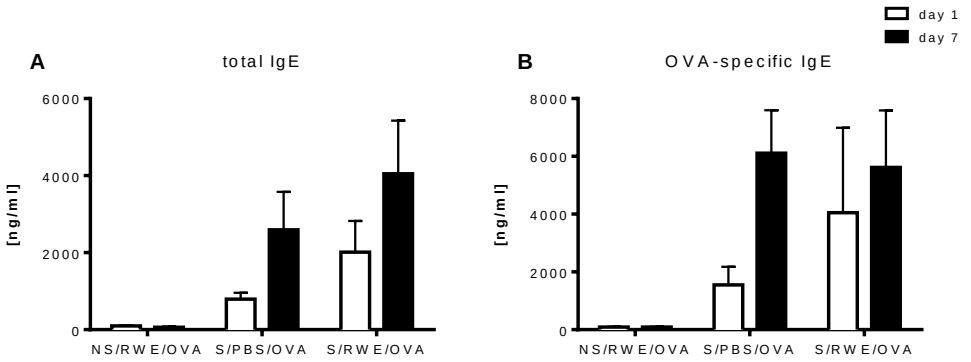

Figure 44: Intranasal instillation of RWE elevates levels of total IgE in plasma of OVA-sensitized mice
Levels of total IgE (A) and OVA-specific IgE (B) in plasma were measured one day (white bars) and seven days (black bars) after the last intranasal Amb-APE instillation/OVA-aerosol challenge.

5 Discussion

5.1 Establishment of a ragweed specific ELISA

To detect ragweed specific IgE and IgG_1 antibodies in the sera of ragweed-sensitized mice an ELISA system was established. ELISAs are a frequently used technique in measuring soluble antigens and antibodies, because of their high sensitivity, the big variety of commercial ELISA kits and the high reproducibility of the assays.

In the first approach for detecting Amb a 1 specific IgE and Amb a 1 specific IgG_1 an sandwich ELISA was established, where microtiter plates were coated directly with purified Amb a 1 and incubated with sera of sensitized mice, enabling binding of specific antibodies to the coated Amb a 1. Afterwards, unbound antibodies were washed away. Bound antibodies were detected by addition of a biotin-conjugated anti-IgE or anti-IgG_1 and a streptavidin-horseradish-peroxidase, which hydrolyzes its substrate TMB. The amount of hydrolyzed substrate is measured photometric and proportional to the amount of bound antibody. Using this indirect ELISA, huge amounts of Amb a 1 specific IgG_1 and well verifiable amounts of Amb a 1 specific IgE could be detected in sera of i.p. sensitized mice. However, in sera of intranasally sensitized mice Amb a 1 specific IgE (but not Amb a 1 specific IgG_1) was below limit of detection. This could be due to the fact that there are many different isotype antibodies in plasma that are specific for Amb a 1 and this therefore bind to the to the immobilized coated antigens. Since the absolute plasma levels of IgG_1 are much higher than the levels of IgE, it is possible that the IgG_1 antibodies displace the IgE antibodies from the binding sites. To avoid the competition between different isotypes, plates were coated with the high affinity receptor for IgE (FcεRI) as a specific "capture antibody" for IgE. As IgE antibodies bind with a very high affinity to the FcεRI ($K_a=10^{10}$ M^{-1}) this approach was thought to be the most sensitive ELISA configuration [167]. For the detection of the bound specific IgE a biotinylated RWE or Amb a 1 was used. Because Amb a 1 was only available in small amounts the direct sandwich ELISA was first tested with biotinylated total RWE. The total RWE is a complex mixture of many different proteins and other substances witch may contain amino groups. Therefore, it is not sure which substances carry the biotin groups after biotinylation and if the free biotin can be removed properly. As there was no specific signal when using sera of ragweed sensitized mice and biotinylated RWE, but a huge unspecific background, biotinylation of total RWE is not the right way. After using biotinylated Amb a 1, we could detect a specific signal in sera of intraperitoneally sensitized mice.

However, even using this very sensitive ELISA approach no Amb a 1 specific IgE was detectable in sera of intranasally sensitized mice. As even the levels of total IgE are very low in this mice compared to i.p. sensitized animals, it can be assumed, that the amount of Amb a 1 specific IgE is under the detection limit.

5.2 Intraperitoneal sensitization in combination with alum leads to a strong Th2 sensitization

5.2.1 Alum triggers the intraperitoneal sensitization

Alum based i.p. immunization protocols are the gold standard for Th2 sensitizing *in vivo*, and a multitude of different studies shows that intraperitoneal sensitization in combination with aluminum hydroxide as adjuvant followed by an intranasal challenge phase leads to a strong Th2-biasd allergic immune response in the lung. In human medicine alum is used as adjuvant in vaccination for more than 80 years, including several childhood vaccines, for example the Diphtheria-Tetanus-Pertussis combination [168]. Surprisingly, there is not much known about the mechanism by which alum exerts its adjuvant potential. In humans alum mainly leads to humoral immune responses, and only induces a weak cell-mediated immunity, which can be measured by delayed type hypersensitivity responses. Different hypotheses for the mechanic effects of alum are discussed. Alum absorbs proteins very well and it has been proposed that alum forms depots, which release the attached antigen slowly, leading to a prolonged immune response with enhanced antibody production. Besides that, by complexation with alum, soluble antigens are converted into a particular form that is phagocytized by antigen presenting cells, like dendritic cells, macrophages, and B cells. By activation of cellular signaling pathways by alum, activation of macrophages, dendritic cells, and antigen presenting cells leads to a shift towards Th2 immune response [155]. Importantly, alum releases uric acid, which is an endogenous danger signal. This leads to the differentiation of monocyte-derived inflammatory dendritic cells, which boost the adaptive immunity [169].

Alum seems to exert a multitude of actions, potentiates the immune response and is used in most models of experimental allergy. Alum is efficiently used combination with different allergens, like ovalbumin [170, 171], house dust mite [172] as well as ragweed [165, 173]. In accordance with literature we saw after intraperitoneal injection of RWE in combination with alum (either on day 0 and 4, or on day or on day 0, day 7, day 14, and day 21) a moderate increase of total IgE, Amb a 1 specific IgE, specific IgG_1 and specific IgG_{2a} in plasma. After a following intranasal challenge phase (either one, two or three challenges) we observed high levels of total IgE, Amb a 1 specific IgE, and Amb a 1 specific IgG_1 in plasma. Additionally, numbers of total cells, lymphocytes, and most of all eosinophils were elevated in BALF and PAS staining of lung sections showed cell infiltration and goblet cell formation. Taken

together, we induced an effective Th2 sensitization by i.p. injection of RWE in combination with alum followed by repeated intranasal challenge with RWE.

5.2.2 The intensity of the intraperitoneal sensitization is strongly dose dependent

It is known from literature that the sensitizing dose is an important factor in defining the outcome of experimental induced allergic diseases. Aguilar-Pimentel et al. showed that in a model of OVA-induced allergic airway inflammation low-dose sensitization (10µg OVA) leads to a significant induction of total and OVA-specific IgE in plasma, eosinic airway inflammation, and high IL-4 levels in BALF, whereas the high dose protocol (10 mg OVA) was characterized by a significant reduction of the allergic phenotype [174]. We showed that after sensitization with a low dose of RWE the levels total IgE, Amb a 1 specific IgE, and Amb a 1 specific IgG_1 reached much higher levels compared to sensitization with a high dose of RWE. In contrast, levels of specific IgG_{2a} were much lower after low-dose sensitization compared to high-dose sensitization. These results fit perfectly with the study of Sakai et al., who shows in a OVA model that after sensitization with a low dose (10 µg OVA) high IgE and low IgG_{2a} titers were found, while low IgE and high IgG_{2a} titers where seen after sensitization with a high dose (1000 µg OVA) [175].

Furthermore, we saw a higher cell infiltration into the BALF after low-dose sensitization, which was mainly due to infiltration of eosinophils and a clear lung inflammation, shown by cell infiltration and goblet cell hyperplasia. This confirmed the study by Sakai et al., who described an inverse correlation between the sensitization dose and the total and eosinophil cell count in BALF. Further they showed that IL-4 and IL-5 are increased after low dose exposure, whereas INF-γ and IL-12 are increased after high dose exposure [175]. The stronger cell infiltration after low dose sensitization compared to high dose sensitization, together with increased levels of total and Amb a 1 specific IgE and Amb a 1 specific IgG_1 and a decreased secretion of IgG_{2a}, suggests that low doses of RWE lead to an Th2 biased immunization, whereas high doses of RWE induce a Th1 immune response.

Besides testing different doses of RWE in sensitization, we also analyzed the influence of the prolongation of the established protocol by extending the sensitization phase form four days to three weeks with four sensitizations instead of two. As expected, this prolongation led to a

stronger sensitization with increased cell infiltration into the BALF, higher levels of Th2 cytokines (IL-4 and IL-5) in the BALF, but only slightly increased titers of specific immunoglobulins in plasma.

5.2.3 Ozone-treatment of ragweed plants has no effect on induction of allergic airway inflammation

Using the established intraperitoneal sensitization protocol, we tested ragweed pollen, grown under different concentrations of ozone according to their effects on elicitation of allergic asthma. Pollen was collected from ragweed plants, which were grown under controlled conditions in climate chambers and exposed to either normal levels of ozone (40 ppb) or elevated ozone levels (80 ppb). Both conditions are realistic concentrations, which can be measured in urban areas. 40 ppb ozone represents the normal level of ozone in ambient air, whereas 80 ppb represents a slightly elevated concentration. On hot summer days levels up to 275 ppb were measured in Germany [176]. In ragweed plants levels of 80 ppb ozone showed now influence on different growth parameters like leaf area, total biomass or catkin weight. Even if there were some non-significant changes in plant size and pollen amount, ragweed plants seem to be insensitive to ozone concentrations up to 80 ppb [97, 99].

For evaluation if the ozone treatment had an influence on the allergenicity in experimental induced asthma, we treated mice with commercial Allergon pollen, control pollen (40 ppb ozone) and pollen grown under elevated ozone conditions (80 ppb ozone) either in the sensitization phase or the election phase of allergic asthma. Although it is known, that ozone can have an influence on pollen viability and induce oxidative stress via NADPH oxidase activity [98], we neither detected an effect in sensitization phase nor in elicitation phase of allergic asthma. There were no differences in immunoglobulin levels or cell infiltration in the lung between the groups treated with pollen grown under high or low ozone conditions. We assume that the sensitization induced by intraperitoneal injection of an allergen in combination with alum is so strong, that it is not possible to distinguish between the minor effects, which could be generated by pollen treated with ozone.

5.2.4 **Conclusion**

Taken together, these experiments show that intraperitoneal ragweed sensitization in combination with alum followed by intranasal ragweed challenge leads to a strong systemic sensitization with a severe allergic reaction in the lung. Interestingly, there is an inverse correlation between the sensitizing dose and the cell count in the BAL fluid. In accordance with other studies, these experiments show that high doses of antigen lead to the production of IgG_{2a}, whereas low antigen doses result in production of IgE and IgG_1 [175, 177]. Although it is known that ambient ozone concentrations can influence the properties of pollen, no difference in sensitization with pollen grown under elevated ozone concentrations compared to sensitization with control pollen was observed in our sensitization model.

5.3 Intranasal sensitization leads to an effective Th2 sensitization

5.3.1 Elicitation of local and systemic Th2 response by intranasal instillation

As intraperitoneal sensitization does not represent the physiological way of pollen exposure and the strong immunization effect may be mainly in consequence of alum, we established an intranasal sensitization protocol, which mimics the natural way by which pollen reach the mucosa. Therefore, we instilled mice on eleven consecutive days with RWE to imitate the chronic exposure to pollen during the pollen season.

Although pollen in common is the most important airborne elicitor of allergy and especially ragweed allergy is known to affect a large percentage of the population in North America and now has gained high significance as neo-allergen in Europe, there is no *in vivo* model of allergic airway responses available that allows to study the sensitizing properties of ragweed via the physiologic mucosal route without the use of artificial adjuvants. There are different models of ragweed allergy, but in most of them ragweed sensitization is caused by intraperitoneal injection of alum (as already discussed) and only the elicitation of allergy is provoked by mucosal allergen administration, for example via the nasal mucosa or the lung epithelium to induce allergic asthma [143, 165] or the eye for induction of allergic conjunctivitis [178]. In one model mice are ragweed sensitized via the nasal mucosa but in combination with GM-CSF as adjuvants [142].

Native pollen often is colonized by microbes and therefore contaminated by lipopolysaccharides (LPS), which is known to be a potent Toll-like receptor 4 (TLR4) ligand and therefore acting as a natural adjuvant for Th2 sensitization [161, 179]. To exclude an adjuvant effect of LPS we used ragweed pollen harvested from plants grown in exposure chambers simulating environmental conditions. These pollen show a very low LPS contamination (<1 EU/ml in the RWE) and it is assumed that the plants were colonized by bacteria only to a very small extent. It was shown already that this at this low dose LPS does not lead to influx of neutrophils into the BAL after daily treatment and therefore LPS should not have confounded the ragweed-specific effects. After 11 intranasal instillations on consecutive days there was a local reaction in the lung, which was shown by elevated numbers of cells in the BALF, increased expression of IL-4 and IL-13 mRNA in the lung, and impaired lung histology with cell infiltration and goblet cell formation. Additionally, there was a systemic Th2 immune reaction in the spleen. After restimulation with RWE or Amb a 1, splenocytes from intranasally ragweed treated mice produced significant elevated levels of

IL-4 and IL-5, but there was no increase of IFN-γ production. Furthermore, restimulation with RWE but not Amb a 1 of splenocytes of sensitized mice (either by intranasal instillation or by i.p. injection) led to increased secretion of IL-10. IL-10 was initially described as a Th2-type cytokine and later as a regulatory cytokine secreted by regulatory T cells (T_{reg}). Today it is clear, that in humans IL-10 is not only secreted by Th2 or T_{reg} cells, but is a much more broadly expressed cytokine, which is produced by many cells of the adaptive immune system, as well as by cells of the innate immune system. It plays an important role as a feedback regulator and controls Th1 as well as Th2 cell responses [180]. However, in mice IL-4 seems to enhance IL-10 mRNA and protein expression, and therefore may be still considered as Th2 dependent cytokine [181]. By enhancing the IL-10 synthesis, IL-4 may promote the differentiation of T_{reg} in mice. As IL-10 production is only seen after restimulation with total RWE, but not with Amb a 1, it could be assumed, that some components of the total extract are able to facilitate T_{reg} responses.

5.3.2 **Biphasic response in instillation time course**

To investigate the kinetic of sensitization induced by intranasal instillation, mice were instilled once, 3 times, 8 times or 11 times with RWE. In this time course experiment we showed that in BALF the total cell count and the number of eosinophils increases with the times of instillations, with highest numbers after 11 intranasal instillations. Neutrophils started to rise already after one instillation, were significantly elevated after 3 instillations, and peaked after 8 instillations. The early influx of neutrophils, followed by an intense influx of eosinophils during elicitation of allergic asthma is shown in different studies after intraperitoneal sensitization with allergen in combination with alum [156, 182] and confirms the development of a strong Th2 immune response starting after eight to eleven days of instillation. This is confirmed by expression of IL-4 and IL-13 mRNA in the lung after eight instillations and by cell infiltration and mucus production by goblet cells in the lung tissue.

5.3.3 A physiological model for allergic pollen sensitization

The model described here enables us to mimic and to dissect the disease mechanisms of allergic sensitization and inflammation triggered by ragweed pollen itself, which initially take place in the exposed tissues and subsequently spread to the lymphoid organs.

Studies on migrants have shown that manifestation of pollinosis like allergic asthma and atopic eczema in most cases requires more than two years, with repeated periods of pollen exposure [183, 184]. However, a ragweed-specific B cell response was detected after 11 days of intranasal instillation of RWE, which corresponds to about half of the duration of a typical birch pollen season, or about one fourth of a ragweed pollen season [70]. Moreover, a strong Th2 immune response and allergic lung inflammation were observed at this time point, reaching similar levels compared with the classical allergen/alum i.p. sensitization protocol. This observation in the allergy-prone BALB/c mouse strain, which serves as a surrogate for susceptible atopic human individuals, provides experimental support for the clinical observation that even adult migrants or local residents, which are exposed to potent neo-allergens, can rapidly develop sensitization upon allergen exposure. Therefore, even after short-term exposure during a period of high pollen counts, early testing for newly developed sensitizations should be considered, when clinical symptoms and allergen exposure are indicative for a neo-sensitization.

5.3.4 Instillation of different compounds of ragweed pollen

For evaluation of the effects of the protein-free fraction of the total RWE, the total extract was fractionated by ultracentrifugation in a protein-free filtrate (>3 kDa) and a protein-containing concentrate (>3 kDa). However, it is important to consider, that some of these small compounds may stick in the filter or remain in the concentrate. As we can be sure that the filtrate (<3 kDa) is definitely free of proteins, but there still may be some of the PALMs in the concentrate (>3 kDa) for the following experiments only the fraction <3 kDa was used. Adenosine, an important component of the protein-free fraction, was shown to be contained exclusively in the protein-free fraction.

Adenosine was removed from the total extract by adenosine deaminase (ADA) digestion. To exclude effects of by treatment, total RWE was sham-digested and used as positive control.

5.3.4.1 Only total RWE elicits sensitizing properties

We analyzed the differential sensitizing impact of intranasal given Amb a 1, protein-free fraction (RWE <3 kDa), and total RWE. Intranasal instillation of total RWE induced a complete allergic airway inflammation, which was characterized by influx of neutrophils, eosinophils, and lymphocytes into the BALF. The airway inflammation induced by instillation of the total extract was also proofed by goblet cell hyperplasia and cell infiltration into the lung tissue. In contrast, instillation of the protein-free fraction (RWE <3 kDa) revealed an isolated proinflammatory influx of neutrophils into the lung.

These data suggest that besides the allergens, which are known to play an important role in sensitization, also low molecular weight substances derived from pollen exert proinflammatory effects *in vivo.* The proinflammatory effect of these low molecular weight substances has been suggested in previous human *in vitro* studies [76, 77]. However, immunoglobulins in plasma were only induced by instillation with total RWE. Neither Amb a 1 allergen alone nor Amb a 1 in combination with the low-molecular weight fraction (RWE <3 kDa) were able to induce ragweed-specific IgG_1 or allergic airway inflammation. These findings suggest that sensitization is elicited mainly by the total extract which contains many different allergens and small molecular weight substances, not by the major allergen Amb a 1 alone. However, small molecules (>3 kDa) also have proinflammatory and adjuvant properties, which can be important for the development of allergic asthma.

Recent studies on mucosal immunity have underlined the importance of epithelium-derived cytokines, such as GM-CSF, TSLP as well as IL-25 and IL-33, which induce pro-allergic innate lymphoid cells (such as nuocytes) in the sensitization phase of atopic diseases [185-188]. Our results suggest that in murine lung tissue after 11 days only, this early phase has been terminated, since the gene-expression of GM-CSF, TSLP, IL-25, IL-33 and proinflammatory cytokines (IL-1β, TNF-α) was not elevated in RWE-instilled mice. Instead, a characteristic Th2-pattern with elevated expression of IL-4 and IL-13 and reduced IFN-γ expression was observed, while B cell activating factors BAFF and APRIL were not regulated.

5.3.4.2 Depletion of adenosine in RWE leads to decreased allergic immune response

Adenosine (ADO) is an endogenous signaling purine nucleoside with many functions in physiological processes. It is a break down product of ATP and can be located intracellular as

well as extracellular. Adenosine has a relatively short biological half live (about 10 seconds) and its degradation is mediated by adenosine deaminase (ADA). Signaling takes place by recognition via four G-protein coupled receptors (A_1, A_{2A}, A_{2B}, and A_3). Under baseline conditions, adenosine concentrations are in nanomolar ranges, but in case of tissue injury or inflammation adenosine levels can increase up to 100fold [189, 190]. Furthermore adenosine was shown to increase the levels of mast cell-released mediators like histamine, tryptase, and prostaglandins. It plays a role in chronic obstructive pulmonary disease and asthma and it is suggested that it is produced in the asthmatic airways where it induces bronchoconstriction [191]. Adenosine deaminase deficient mice, which have elevated levels of adenosine showed pulmonary inflammation with infiltration of eosinophils, mucus hyper secretion and airway obstruction. These symptoms could be reversed by lowering the adenosine concentration in the lung using ADA enzyme therapy [192]. Recently it was shown that extracellular adenosine regulates early thymic T cell development and is important for the maintenance to sustain normal numbers of naïve T cell in the periphery [193].

The low molecular weight fraction of aqueous birch pollen extract, but also of other pollen species, was shown to contain micromolar concentrations of adenosine and this pollen-associated adenosine contributes substantially to the modulation of DC function, like inhibition of T helper cell responses and induction of T_{reg} cells [80]. The adenosine content of ragweed extract used for the intranasal installation was measured by ULPC (ultra performance liquid chromatography) and determined as 0.0327 mM. To evaluate the effects of adenosine within this whole extract, mice were treated with either the total extract (which contains adenosine) or with an extract with was depleted of adenosine by adenosine-deaminase (ADA) treatment. In order to exclude an effect of the ADA-treatment, the total extract was sham-digested and processed in the same way like the ADA-digested extract. Mice treated 11 times intranasally with the extract depleted of adenosine had decreased levels of lymphocytes, eosinophils, and neutrophils in the lung and significant lower levels of Amb a 1 specific IgG_1 in plasma. Furthermore, depletion of adenosine leads to a decreased production of Th2 cytokines by splenocytes and cells of cervical lymph nodes after restimulation by RWE or Amb a 1. The reduced cell infiltration in the lung and also the decreased systemic Th2 response indicate that pollen-associated adenosine seems to play an important role in elicitation of pollen-induced allergic asthma. As adenosine is present in many different pollen species (*Betula, Phleum, Pinus, and Ambrosia*) [80], it could be an important adjuvant factor in elicitation of different pollen allergies.

Although we could show no effect of the instillation of adenosine alone, it was shown before that adenosine challenge increased the recruitment of eosinophils and neutrophils into the BALF in sensitized and challenged mice [144].

5.3.5 Bystander effect of intranasal RWE instillation on OVA-induced airway inflammation

Previously, it was shown that proinflammatory stimuli, such as ultrafine carbon particles, generate a proinflammatory micro milieu, which substantially augments allergic airway inflammation during subsequent allergen-exposure [194]. This adjuvant activity was shown to be mediated by reactive oxygen species, which cause oxidative stress in the lung [170, 195]. In this context, the intranasal instillation of RWE for 11 days in OVA-sensitized mice substantially exacerbated the otherwise mild and only transient allergic inflammation which follows a single OVA-aerosol challenge. Intranasal instillation of RWE led to a persistent inflammatory lung infiltrate with strongly increased mucus production in OVA-pre-sensitized mice that was still present after seven days. The exact mechanism by which RWE augments OVA-specific inflammation is still elusive, but the presence of reactive oxygen species can be assumed, due to pollen-derived NADPH-oxidases [73]. Because intranasal RWE-administration by itself already induced a transient allergic airway inflammation, adjuvant effects of RWE on a pre-existing bystander sensitization can by assumed. There is an synergism between the OVA-induced and the ragweed-induced inflammation, in which ragweed provides unspecific as well as specific proinflammatory components. This finding provides an experimental support for the increased symptom scores observed in patients with polysensitization against ragweed plus *Betulaceae*, *Poaceae* and *Artemisia*, compared to patients with ragweed mono-sensitization [196]. In this context it should be considered, that ragweed allergy could especially affect people who already suffer from a preexisting allergy or sensitization.

5.3.6 Conclusion

Taken together, these data show the generation of an *in vivo* model that mimics physiological exposure of the airways with ragweed as a potent environmentally relevant agent. We demonstrated that as early as 11 days after intranasal allergen exposure, clinically relevant T and B cell sensitization can be induced and allergic airway inflammation elicited. Adenosine

was shown to play an important adjuvant role in sensitization towards ragweed extract. Moreover, ragweed pollen exerted proinflammatory effects, and RWE-induced lung inflammation augmented subsequent allergic reactions towards an unrelated allergen (OVA). These data provide evidence that even short-term exposure to potent allergen sources can induce clinically relevant *de-novo* sensitizations and aggravate existing bystander-allergy. We currently use this model to decipher, which pollen-derived mediators are responsible for proinflammatory and sensitizing properties of pollen and thus help to define novel drug targets for treatment of respiratory allergies. Further studies should aim on therapeutic strategies to control allergen-depended as well as allergen-independent pathways to prevent sensitization or mediate tolerance towards ragweed.

6 Literature

1. Pirquet, C.v., *Allergie.* Münchner Medizinische Wochenschrift, 1906. **30**: p. 1457-1458.
2. Ring, J., *Angewandte Allergologie*. Auflage: 3. ed2003: Urban und Vogel.
3. Kay, A.B., *100 years of 'Allergy': can von Pirquet's word be rescued?* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2006. **36**(5): p. 555-9.
4. Durham, S.R., et al., *Immunologic studies in allergen-induced late-phase asthmatic reactions.* The Journal of allergy and clinical immunology, 1984. **74**(1): p. 49-60.
5. Cieslewicz, G., et al., *The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration.* The Journal of clinical investigation, 1999. **104**(3): p. 301-8.
6. Sleiman, P.M., et al., *Variants of DENND1B associated with asthma in children.* N Engl J Med, 2010. **362**(1): p. 36-44.
7. Kurzius-Spencer, M., et al., *Familial aggregation of allergen-specific sensitization and asthma.* Pediatr Allergy Immunol, 2012. **23**(1): p. 21-7.
8. Galli, S.J., M. Tsai, and A.M. Piliponsky, *The development of allergic inflammation.* Nature, 2008. **454**(7203): p. 445-54.
9. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* Journal of immunology, 1986. **136**(7): p. 2348-57.
10. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nat Immunol, 2005. **6**(11): p. 1133-41.
11. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* Journal of immunology, 1995. **155**(3): p. 1151-64.
12. Veldhoen, M., et al., *Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset.* Nat Immunol, 2008. **9**(12): p. 1341-6.
13. Eyerich, S., et al., *Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling.* The Journal of clinical investigation, 2009. **119**(12): p. 3573-85.
14. King, I.L. and M. Mohrs, *IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells.* The Journal of experimental medicine, 2009. **206**(5): p. 1001-7.
15. Jahnsen, F.L., et al., *Accelerated antigen sampling and transport by airway mucosal dendritic cells following inhalation of a bacterial stimulus.* Journal of immunology, 2006. **177**(9): p. 5861-7.
16. Xiao, C., et al., *Defective epithelial barrier function in asthma.* The Journal of allergy and clinical immunology, 2011. **128**(3): p. 549-56 e1-12.
17. Kauffman, H.F., et al., *House dust mite major allergens Der p 1 and Der p 5 activate human airway-derived epithelial cells by protease-dependent and protease-independent mechanisms.* Clin Mol Allergy, 2006. **4**: p. 5.
18. Jeong, S.K., et al., *Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery.* J Invest Dermatol, 2008. **128**(8): p. 1930-9.
19. Runswick, S., et al., *Pollen proteolytic enzymes degrade tight junctions.* Respirology, 2007. **12**(6): p. 834-42.

20. Reinherz, E.L., et al., *The crystal structure of a T cell receptor in complex with peptide and MHC class II.* Science, 1999. **286**(5446): p. 1913-21.
21. Yoshimoto, T., et al., *Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells.* Nat Immunol, 2009. **10**(7): p. 706-12.
22. Wang, M., A. Saxon, and D. Diaz-Sanchez, *Early IL-4 production driving Th2 differentiation in a human in vivo allergic model is mast cell derived.* Clin Immunol, 1999. **90**(1): p. 47-54.
23. Zhang, D.H., et al., *Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene.* The Journal of biological chemistry, 1997. **272**(34): p. 21597-603.
24. Kurata, H., et al., *Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells.* Immunity, 1999. **11**(6): p. 677-88.
25. Yamane, H., J. Zhu, and W.E. Paul, *Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment.* The Journal of experimental medicine, 2005. **202**(6): p. 793-804.
26. Zhu, J. and W.E. Paul, *Heterogeneity and plasticity of T helper cells.* Cell Res, 2010. **20**(1): p. 4-12.
27. Amsen, D., et al., *Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch.* Immunity, 2007. **27**(1): p. 89-99.
28. Le Gros, G., et al., *Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells.* The Journal of experimental medicine, 1990. **172**(3): p. 921-9.
29. Longphre, M., et al., *Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells.* The Journal of clinical investigation, 1999. **104**(10): p. 1375-82.
30. Fort, M.M., et al., *IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo.* Immunity, 2001. **15**(6): p. 985-95.
31. Mathur, M., et al., *CD28 interactions with either CD80 or CD86 are sufficient to induce allergic airway inflammation in mice.* Am J Respir Cell Mol Biol, 1999. **21**(4): p. 498-509.
32. Spriggs, M.K., et al., *Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion.* The Journal of experimental medicine, 1992. **176**(6): p. 1543-50.
33. Gould, H.J. and B.J. Sutton, *IgE in allergy and asthma today.* Nature reviews. Immunology, 2008. **8**(3): p. 205-17.
34. Yancopoulos, G.D. and F.W. Alt, *Developmentally controlled and tissue-specific expression of unrearranged VH gene segments.* Cell, 1985. **40**(2): p. 271-81.
35. Jones, J.M. and M. Gellert, *Ordered assembly of the V(D)J synaptic complex ensures accurate recombination.* EMBO J, 2002. **21**(15): p. 4162-71.
36. Wesemann, D.R., et al., *Immature B cells preferentially switch to IgE with increased direct Smu to Sepsilon recombination.* The Journal of experimental medicine, 2011. **208**(13): p. 2733-46.
37. Muramatsu, M., et al., *Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells.* The Journal of biological chemistry, 1999. **274**(26): p. 18470-6.
38. Xu, Z., et al., *Immunoglobulin class-switch DNA recombination: induction, targeting and beyond.* Nature reviews. Immunology, 2012. **12**(7): p. 517-31.
39. Humbert, M., et al., *High-affinity IgE receptor (FcepsilonRI)-bearing cells in bronchial biopsies from atopic and nonatopic asthma.* American journal of respiratory and critical care medicine, 1996. **153**(6 Pt 1): p. 1931-7.

40. Sihra, B.S., et al., *Expression of high-affinity IgE receptors (Fc epsilon RI) on peripheral blood basophils, monocytes, and eosinophils in atopic and nonatopic subjects: relationship to total serum IgE concentrations.* The Journal of allergy and clinical immunology, 1997. **99**(5): p. 699-706.
41. Lindau, M. and J.M. Fernandez, *IgE-mediated degranulation of mast cells does not require opening of ion channels.* Nature, 1986. **319**(6049): p. 150-3.
42. Abul Abbas, A.L., Shiv Pillai, *Cellular and Molecular Immunology*. 7 ed2007: Saunders.
43. Ringvall, M., et al., *Serotonin and histamine storage in mast cell secretory granules is dependent on serglycin proteoglycan.* The Journal of allergy and clinical immunology, 2008. **121**(4): p. 1020-6.
44. Merickel, A. and R.H. Edwards, *Transport of histamine by vesicular monoamine transporter-2.* Neuropharmacology, 1995. **34**(11): p. 1543-7.
45. Schwartz, L.B., et al., *Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells.* Journal of immunology, 1987. **138**(8): p. 2611-5.
46. Waern, I., et al., *Mast cells limit extracellular levels of IL-13 via a serglycin proteoglycan-serine protease axis.* Biol Chem, 2012.
47. Peters, S.P., et al., *Arachidonic acid metabolism in purified human lung mast cells.* Journal of immunology, 1984. **132**(4): p. 1972-9.
48. Ohkawara, Y., et al., *Human lung mast cells and pulmonary macrophages produce tumor necrosis factor-alpha in sensitized lung tissue after IgE receptor triggering.* Am J Respir Cell Mol Biol, 1992. **7**(4): p. 385-92.
49. Bradding, P., et al., *TNF alpha is localized to nasal mucosal mast cells and is released in acute allergic rhinitis.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 1995. **25**(5): p. 406-15.
50. Feuser, K., et al., *Human intestinal mast cells are a potent source of multiple chemokines.* Cytokine, 2012. **58**(2): p. 178-85.
51. Holgate, S.T., *Epithelium dysfunction in asthma.* The Journal of allergy and clinical immunology, 2007. **120**(6): p. 1233-44; quiz 1245-6.
52. Benayoun, L., et al., *Airway structural alterations selectively associated with severe asthma.* American journal of respiratory and critical care medicine, 2003. **167**(10): p. 1360-8.
53. Molet, S., et al., *IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines.* The Journal of allergy and clinical immunology, 2001. **108**(3): p. 430-8.
54. Pennino, D., et al., *IL-22 suppresses IFN-gamma-mediated lung inflammation in asthmatic patients.* The Journal of allergy and clinical immunology, 2013. **131**(2): p. 562-70.
55. Kabesch, M., et al., *A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood.* The Journal of allergy and clinical immunology, 2003. **112**(5): p. 893-8.
56. Chavanas, S., et al., *Mutations in SPINK5, encoding a serine protease inhibitor, cause Netherton syndrome.* Nat Genet, 2000. **25**(2): p. 141-2.
57. Sayers, I., et al., *Allelic association and functional studies of promoter polymorphism in the leukotriene C4 synthase gene (LTC4S) in asthma.* Thorax, 2003. **58**(5): p. 417-24.
58. Simpson, A., et al., *Polymorphisms in a disintegrin and metalloprotease 33 (ADAM33) predict impaired early-life lung function.* American journal of respiratory and critical care medicine, 2005. **172**(1): p. 55-60.
59. Gilliland, F.D., et al., *Effects of glutathione S-transferase M1, maternal smoking during pregnancy, and environmental tobacco smoke on asthma and wheezing in children.* American journal of respiratory and critical care medicine, 2002. **166**(4): p. 457-63.

60. Mapp, C.E., et al., *Asthma induced by isocyanates: a model of IgE-independent asthma.* Acta Biomed, 2005. **76 Suppl 2**: p. 15-9.
61. Balzar, S., et al., *Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program.* American journal of respiratory and critical care medicine, 2011. **183**(3): p. 299-309.
62. Bumbacea, D., et al., *Parameters associated with persistent airflow obstruction in chronic severe asthma.* Eur Respir J, 2004. **24**(1): p. 122-8.
63. Holgate, S.T., et al., *A new look at the pathogenesis of asthma.* Clin Sci (Lond), 2010. **118**(7): p. 439-50.
64. Gern, J.E., et al., *Relationship of upper and lower airway cytokines to outcome of experimental rhinovirus infection.* American journal of respiratory and critical care medicine, 2000. **162**(6): p. 2226-31.
65. Grunberg, K., et al., *Effect of experimental rhinovirus 16 colds on airway hyperresponsiveness to histamine and interleukin-8 in nasal lavage in asthmatic subjects in vivo.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 1997. **27**(1): p. 36-45.
66. Edlund, A.F., R. Swanson, and D. Preuss, *Pollen and stigma structure and function: the role of diversity in pollination.* Plant Cell, 2004. **16 Suppl**: p. S84-97.
67. Aalberse, R.C., *Structural biology of allergens.* The Journal of allergy and clinical immunology, 2000. **106**(2): p. 228-38.
68. Gilles, S., et al., *The pollen enigma: modulation of the allergic immune response by non-allergenic, pollen-derived compounds.* Curr Pharm Des, 2012. **18**(16): p. 2314-9.
69. Buters, J.T., et al., *Year-to-year variation in release of Bet v 1 allergen from birch pollen: evidence for geographical differences between West and South Germany.* International archives of allergy and immunology, 2008. **145**(2): p. 122-30.
70. Buters, J.T., et al., *The allergen Bet v 1 in fractions of ambient air deviates from birch pollen counts.* Allergy, 2010. **65**(7): p. 850-8.
71. Frahry, G. and P. Schopfer, *NADH-stimulated, cyanide-resistant superoxide production in maize coleoptiles analyzed with a tetrazolium-based assay.* Planta, 2001. **212**(2): p. 175-83.
72. Foreman, J., et al., *Reactive oxygen species produced by NADPH oxidase regulate plant cell growth.* Nature, 2003. **422**(6930): p. 442-6.
73. Boldogh, I., et al., *ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation.* The Journal of clinical investigation, 2005. **115**(8): p. 2169-79.
74. Wolters-Arts, M., W.M. Lush, and C. Mariani, *Lipids are required for directional pollen-tube growth.* Nature, 1998. **392**(6678): p. 818-21.
75. Behrendt, H., et al., *Secretion of proinflammatory eicosanoid-like substances precedes allergen release from pollen grains in the initiation of allergic sensitization.* International archives of allergy and immunology, 2001. **124**(1-3): p. 121-5.
76. Traidl-Hoffmann, C., et al., *Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes.* The Journal of allergy and clinical immunology, 2002. **109**(5): p. 831-8.
77. Plotz, S.G., et al., *Chemotaxis and activation of human peripheral blood eosinophils induced by pollen-associated lipid mediators.* The Journal of allergy and clinical immunology, 2004. **113**(6): p. 1152-60.
78. Traidl-Hoffmann, C., et al., *Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization.* The Journal of experimental medicine, 2005. **201**(4): p. 627-36.

79. Gutermuth, J., et al., *Immunomodulatory effects of aqueous birch pollen extracts and phytoprostanes on primary immune responses in vivo.* The Journal of allergy and clinical immunology, 2007. **120**(2): p. 293-9.
80. Gilles, S., et al., *Pollen metabolome analysis reveals adenosine as a major regulator of dendritic cell-primed T(H) cell responses.* The Journal of allergy and clinical immunology, 2011. **127**(2): p. 454-461 e1-9.
81. Alberternst, B., S. Nawrath, and F. Klingenstein, *Biology, distribution and pathways of introduction of Ambrosia artemisiifolia in Germany and assessment from a nature conservation point of view.* Nachrichtenblatt des Deutschen Pflanzenschutzdienstes, 2006. **58**: p. 279-285.
82. Cecchi, L., et al., *Long distance transport of ragweed pollen as a potential cause of allergy in central Italy.* Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology, 2006. **96**(1): p. 86-91.
83. Breton, M.-C., et al., *Relationship between climate, pollen concentrations of Ambrosia and medical consultations for allergic rhinitis in Montreal, 1994-2002.* Science of The Total Environment, 2006. **370**: p. 39-50.
84. Hauser, M., et al., *Panallergens and their impact on the allergic patient.* Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology, 2010. **6**(1): p. 1.
85. Leonard, R., et al., *A new allergen from ragweed (Ambrosia artemisiifolia) with homology to art v 1 from mugwort.* The Journal of biological chemistry, 2010. **285**(35): p. 27192-200.
86. Oberhuber, C., et al., *Prevalence of IgE-binding to Art v 1, Art v 4 and Amb a 1 in mugwort-allergic patients.* International archives of allergy and immunology, 2008. **145**(2): p. 94-101.
87. Goodfriend, L., et al., *Two variants of ragweed allergen Ra3.* The Journal of allergy and clinical immunology, 1981. **67**(4): p. 299-304.
88. Roebber, M., D.G. Klapper, and D.G. Marsh, *Two isoallergens of short ragweed component Ra5.* Journal of immunology, 1982. **129**(1): p. 120-5.
89. Marzban, G., et al., *Fruit cross-reactive allergens: a theme of uprising interest for consumers' health.* Biofactors, 2005. **23**(4): p. 235-41.
90. Hiller, K.M., B.C. Lubahn, and D.G. Klapper, *Cloning and expression of ragweed allergen Amb a 6.* Scand J Immunol, 1998. **48**(1): p. 26-36.
91. Hauser, M., et al., *Molecular Properties of Plant Food Allergens: A Current Classification into Protein Families.* The Open Immunology Journal, 2008(1): p. 1-12.
92. Asero, R., G. Mistrello, and S. Amato, *The nature of melon allergy in ragweed-allergic subjects: A study of 1000 patients.* Allergy Asthma Proc, 2011. **32**(1): p. 64-7.
93. Wopfner, N., et al., *Molecular and immunological characterization of novel weed pollen pan-allergens.* Allergy, 2008. **63**(7): p. 872-81.
94. Ziska, L.H., P.R. Epstein, and W.H. Schlesinger, *Rising CO(2), climate change, and public health: exploring the links to plant biology.* Environ Health Perspect, 2009. **117**(2): p. 155-8.
95. Wayne, P., et al., *Production of allergenic pollen by ragweed (Ambrosia artemisiifolia L.) is increased in CO2-enriched atmospheres.* Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology, 2002. **88**(3): p. 279-82.
96. Ziska, L.H., et al., *Cities as harbingers of climate change: common ragweed, urbanization, and public health.* The Journal of allergy and clinical immunology, 2003. **111**(2): p. 290-5.
97. Ziska, L.H., *Sensitivity of ragweed (Ambrosia artemisiifolia) growth to urban ozone concentrations.* Functional Plant Biology, 2002. **29**: p. 1365-1369.

98. Pasqualini, S., et al., *Ozone affects pollen viability and NAD(P)H oxidase release from Ambrosia artemisiifolia pollen.* Environ Pollut, 2011. **159**(10): p. 2823-30.
99. Kanter, U., et al., *Molecular and immunological characterization of ragweed (Ambrosia artemisiifolia L.) pollen after exposure of the plants to elevated ozone over a whole growing season.* PloS one, 2013. **8**(4): p. e61518.
100. Bjorksten, B., et al., *Worldwide time trends for symptoms of rhinitis and conjunctivitis: Phase III of the International Study of Asthma and Allergies in Childhood.* Pediatr Allergy Immunol, 2008. **19**(2): p. 110-24.
101. Boulet, L.P., et al., *Comparative degree and type of sensitization to common indoor and outdoor allergens in subjects with allergic rhinitis and/or asthma.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 1997. **27**(1): p. 52-9.
102. Arbes, S.J., Jr., et al., *Prevalences of positive skin test responses to 10 common allergens in the US population: results from the third National Health and Nutrition Examination Survey.* The Journal of allergy and clinical immunology, 2005. **116**(2): p. 377-83.
103. Bousquet, P.J., et al., *Geographical variation in the prevalence of positive skin tests to environmental aeroallergens in the European Community Respiratory Health Survey I.* Allergy, 2007. **62**(3): p. 301-9.
104. Alberternst, B. and S. Nawrath, *Spreading and distribution of common ragweed (Ambrosia artemisiifolia) in Germany*, in *Proceedings of the 4th European Symposium of Aerobiology*2008: Turku.
105. Boehme, M.W., et al., *[Sensitization to airborne ragweed pollen--a cause of allergic respiratory diseases in Germany?].* Dtsch Med Wochenschr, 2009. **134**(28-29): p. 1457-63.
106. Rueff, F., et al., *Sensitization to common ragweed in southern Bavaria: clinical and geographical risk factors in atopic patients.* International archives of allergy and immunology, 2012. **159**(1): p. 65-74.
107. Brightling, C.E., et al., *Lung damage and airway remodelling in severe asthma.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2012. **42**(5): p. 638-49.
108. Boyce, J.A., et al., *Advances in mechanisms of asthma, allergy, and immunology in 2011.* The Journal of allergy and clinical immunology, 2012. **129**(2): p. 335-41.
109. Slager, R.E., et al., *Genetics of asthma susceptibility and severity.* Clin Chest Med, 2012. **33**(3): p. 431-43.
110. Gras, D., et al., *An ex vivo model of severe asthma using reconstituted human bronchial epithelium.* The Journal of allergy and clinical immunology, 2012. **129**(5): p. 1259-1266 e1.
111. Howarth, P.H., et al., *Synthetic responses in airway smooth muscle.* The Journal of allergy and clinical immunology, 2004. **114**(2 Suppl): p. S32-50.
112. Chan, V., et al., *Extracellular matrix regulates enhanced eotaxin expression in asthmatic airway smooth muscle cells.* American journal of respiratory and critical care medicine, 2006. **174**(4): p. 379-85.
113. Epstein, M.M., *Do mouse models of allergic asthma mimic clinical disease?* International archives of allergy and immunology, 2004. **133**(1): p. 84-100.
114. Taube, C., A. Dakhama, and E.W. Gelfand, *Insights into the pathogenesis of asthma utilizing murine models.* International archives of allergy and immunology, 2004. **135**(2): p. 173-86.
115. Gelfand, E.W., *Pro: mice are a good model of human airway disease.* American journal of respiratory and critical care medicine, 2002. **166**(1): p. 5-6; discussion 7-8.
116. Persson, C.G., *Con: mice are not a good model of human airway disease.* American journal of respiratory and critical care medicine, 2002. **166**(1): p. 6-7; discussion 8.

117. Richards, I.M., *Mouse models of allergic disease; how do they relate to asthma in man?* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 1996. **26**(6): p. 618-20.
118. Persson, C.G., et al., *The mouse trap.* Trends Pharmacol Sci, 1997. **18**(12): p. 465-7.
119. Kumar, R.K. and P.S. Foster, *Modeling allergic asthma in mice: pitfalls and opportunities.* Am J Respir Cell Mol Biol, 2002. **27**(3): p. 267-72.
120. Pabst, R., *Animal models for asthma: controversial aspects and unsolved problems.* Pathobiology, 2002. **70**(5): p. 252-4.
121. Lloyd, C.M., *Building better mouse models of asthma.* Curr Allergy Asthma Rep, 2007. **7**(3): p. 231-6.
122. Boyce, J.A. and K.F. Austen, *No audible wheezing: nuggets and conundrums from mouse asthma models.* The Journal of experimental medicine, 2005. **201**(12): p. 1869-73.
123. Zosky, G.R. and P.D. Sly, *Animal models of asthma.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2007. **37**(7): p. 973-88.
124. Nials, A.T. and S. Uddin, *Mouse models of allergic asthma: acute and chronic allergen challenge.* Dis Model Mech, 2008. **1**(4-5): p. 213-20.
125. Dietrich, W.F., et al., *A comprehensive genetic map of the mouse genome.* Nature, 1996. **380**(6570): p. 149-52.
126. Stamatoyannopoulos, J.A., et al., *An encyclopedia of mouse DNA elements (Mouse ENCODE).* Genome Biol, 2012. **13**(8): p. 418.
127. Austin, C.P., et al., *The knockout mouse project.* Nat Genet, 2004. **36**(9): p. 921-4.
128. Cherwinski, H.M., et al., *Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies.* The Journal of experimental medicine, 1987. **166**(5): p. 1229-44.
129. Pauwels, R.A., G.J. Brusselle, and J.C. Kips, *Cytokine manipulation in animal models of asthma.* American journal of respiratory and critical care medicine, 1997. **156**(4 Pt 2): p. S78-81.
130. Foster, P.S., et al., *Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model.* The Journal of experimental medicine, 1996. **183**(1): p. 195-201.
131. Fish, S.C., et al., *IgE generation and mast cell effector function in mice deficient in IL-4 and IL-13.* Journal of immunology, 2005. **174**(12): p. 7716-24.
132. Kumar, R.K., et al., *Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma.* J Pharmacol Exp Ther, 2003. **307**(1): p. 349-55.
133. Coyle, A.J., et al., *Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody.* The Journal of experimental medicine, 1996. **183**(4): p. 1303-10.
134. Henderson, W.R., Jr., E.Y. Chi, and C.R. Maliszewski, *Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma.* Journal of immunology, 2000. **164**(2): p. 1086-95.
135. Leigh, R., et al., *T-cell-mediated inflammation does not contribute to the maintenance of airway dysfunction in mice.* J Appl Physiol, 2004. **97**(6): p. 2258-65.
136. Cates, E.C., et al., *Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism.* Journal of immunology, 2004. **173**(10): p. 6384-92.
137. Fattouh, R., et al., *House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation.* American journal of respiratory and critical care medicine, 2005. **172**(3): p. 314-21.

138. Natarajan, S., et al., *Pulmonary endotoxin tolerance protects against cockroach allergen-induced asthma-like inflammation in a mouse model.* International archives of allergy and immunology, 2012. **158**(2): p. 120-30.
139. Vaickus, L.J., et al., *Cockroach allergens induce biphasic asthma-like pulmonary inflammation in outbred mice.* J Asthma, 2012. **49**(5): p. 510-21.
140. Sharma, S.K., et al., *Systemic FasL neutralization increases eosinophilic inflammation in a mouse model of asthma.* Allergy, 2012. **67**(3): p. 328-335.
141. Kierstein, S., et al., *Ozone inhalation induces exacerbation of eosinophilic airway inflammation and hyperresponsiveness in allergen-sensitized mice.* Allergy, 2008. **63**(4): p. 438-46.
142. Cates, E.C., et al., *Effect of GM-CSF on immune, inflammatory, and clinical responses to ragweed in a novel mouse model of mucosal sensitization.* The Journal of allergy and clinical immunology, 2003. **111**(5): p. 1076-86.
143. Sur, S., et al., *Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides.* Journal of immunology, 1999. **162**(10): p. 6284-93.
144. Fan, M. and S. Jamal Mustafa, *Role of adenosine in airway inflammation in an allergic mouse model of asthma.* Int Immunopharmacol, 2006. **6**(1): p. 36-45.
145. Corry, D.B., et al., *Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity.* The Journal of experimental medicine, 1996. **183**(1): p. 109-17.
146. Shinagawa, K. and M. Kojima, *Mouse model of airway remodeling: strain differences.* American journal of respiratory and critical care medicine, 2003. **168**(8): p. 959-67.
147. Botting, J., *The History of Thalidomide.* Drug News Perspect, 2002. **15**(9): p. 604-611.
148. Bakacs, T., J.N. Mehrishi, and R.W. Moss, *Ipilimumab (Yervoy) and the TGN1412 catastrophe.* Immunobiology, 2012. **217**(6): p. 583-9.
149. Schlesinger, R.B. and L.A. McFadden, *Comparative morphometry of the upper bronchial tree in six mammalian species.* Anat Rec, 1981. **199**(1): p. 99-108.
150. Plopper, C.G., et al., *Comparison of nonciliated tracheal epithelial cells in six mammalian species: ultrastructure and population densities.* Exp Lung Res, 1983. **5**(4): p. 281-94.
151. Uhm, T.G., B.S. Kim, and I.Y. Chung, *Eosinophil development, regulation of eosinophil-specific genes, and role of eosinophils in the pathogenesis of asthma.* Allergy Asthma Immunol Res, 2012. **4**(2): p. 68-79.
152. Wenzel, S., *Severe asthma: from characteristics to phenotypes to endotypes.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2012. **42**(5): p. 650-8.
153. Szelenyi, I., *Animal models of bronchial asthma.* Inflamm Res, 2000. **49**(12): p. 639-54.
154. Kips, J.C., et al., *Murine models of asthma.* Eur Respir J, 2003. **22**(2): p. 374-82.
155. Kool, M., K. Fierens, and B.N. Lambrecht, *Alum adjuvant: some of the tricks of the oldest adjuvant.* J Med Microbiol, 2012. **61**(Pt 7): p. 927-34.
156. Schneider, T., et al., *Kinetics and quantitation of eosinophil and neutrophil recruitment to allergic lung inflammation in a brown Norway rat model.* Am J Respir Cell Mol Biol, 1997. **17**(6): p. 702-12.
157. Underwood, S.L., et al., *IgE production, antigen-induced airway inflammation and airway hyperreactivity in the brown Norway rat: the effects of ricin.* Immunology, 1995. **85**(2): p. 256-61.
158. Tulic, M.K., P.G. Holt, and P.D. Sly, *Modification of acute and late-phase allergic responses to ovalbumin with lipopolysaccharide.* International archives of allergy and immunology, 2002. **129**(2): p. 119-28.

159. Akahira-Azuma, M., et al., *Early delayed-type hypersensitivity eosinophil infiltrates depend on T helper 2 cytokines and interferon-gamma via CXCR3 chemokines.* Immunology, 2004. **111**(3): p. 306-17.
160. Farraj, A.K., et al., *Immune responses in the lung and local lymph node of A/J mice to intranasal sensitization and challenge with adjuvant-free ovalbumin.* Toxicol Pathol, 2003. **31**(4): p. 432-47.
161. Eisenbarth, S.C., et al., *Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen.* The Journal of experimental medicine, 2002. **196**(12): p. 1645-51.
162. Johnson, J.R., et al., *Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling.* American journal of respiratory and critical care medicine, 2004. **169**(3): p. 378-85.
163. Post, S., et al., *The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation.* Thorax, 2012. **67**(6): p. 488-95.
164. Thiel, S., et al., *A phytotron for plant stress research: How far can artificial lighting compare to natural sunlight?* Journal of plant physiology, 1996. **148**(3-4): p. 456-463.
165. Dharajiya, N., et al., *FcgammaRIIb inhibits allergic lung inflammation in a murine model of allergic asthma.* PloS one, 2010. **5**(2): p. e9337.
166. Wopfner, N., et al., *The spectrum of allergens in ragweed and mugwort pollen.* International archives of allergy and immunology, 2005. **138**(4): p. 337-46.
167. Ravetch, J.V. and J.P. Kinet, *Fc receptors.* Annu Rev Immunol, 1991. **9**: p. 457-92.
168. Naim, J.O., et al., *Mechanisms of adjuvancy: I--Metal oxides as adjuvants.* Vaccine, 1997. **15**(11): p. 1183-93.
169. Kool, M., et al., *Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells.* The Journal of experimental medicine, 2008. **205**(4): p. 869-82.
170. Alessandrini, F., et al., *Role of oxidative stress in ultrafine particle-induced exacerbation of allergic lung inflammation.* American journal of respiratory and critical care medicine, 2009. **179**(11): p. 984-91.
171. Lee, T., et al., *Grape seed proanthocyanidin extract attenuates allergic inflammation in murine models of asthma.* J Clin Immunol, 2012. **32**(6): p. 1292-304.
172. Strong, P., et al., *A recombinant fragment of human SP-D reduces allergic responses in mice sensitized to house dust mite allergens.* Clin Exp Immunol, 2003. **134**(2): p. 181-7.
173. Nemeth, K., et al., *Bone marrow stromal cells use TGF-beta to suppress allergic responses in a mouse model of ragweed-induced asthma.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(12): p. 5652-7.
174. Aguilar-Pimentel, J.A., et al., *Specific CD8 T cells in IgE-mediated allergy correlate with allergen dose and allergic phenotype.* American journal of respiratory and critical care medicine, 2010. **181**(1): p. 7-16.
175. Sakai, K., et al., *Effect of different sensitizing doses of antigen in a murine model of atopic asthma.* Clin Exp Immunol, 1999. **118**(1): p. 9-15.
176. Galler, *Einfluss von Ozon auf den Allergengehalt von Graspollen*, in *Paderborn: Universität Paderborn* 2003.
177. Kolbe, L., C. Heusser, and E. Kolsch, *Epitope-dependent nonreciprocal regulation of IgE and IgG2a antibody formation.* International archives of allergy and immunology, 1994. **103**(2): p. 214-6.
178. Li, D.Q., et al., *Short ragweed pollen triggers allergic inflammation through Toll-like receptor 4-dependent thymic stromal lymphopoietin/OX40 ligand/OX40 signaling pathways.* The Journal of allergy and clinical immunology, 2011. **128**(6): p. 1318-1325 e2.

179. Heydenreich, B., et al., *Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2011.
180. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells.* Nature reviews. Immunology, 2010. **10**(3): p. 170-81.
181. Schmidt-Weber, C.B., et al., *IL-4 enhances IL-10 gene expression in murine Th2 cells in the absence of TCR engagement.* Journal of immunology, 1999. **162**(1): p. 238-44.
182. El-Hashim, A.Z., D. Wyss, and C. Zuany-Amorim, *Kinetics of airway hyperresponsiveness and airway eosinophilia in BALB/c mice and their modulation by different dexamethasone treatment regimens.* Pulm Pharmacol Ther, 2002. **15**(5): p. 467-75.
183. Lombardi, C., G.W. Canonica, and G. Passalacqua, *The possible influence of the environment on respiratory allergy: a survey on immigrants to Italy.* Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology, 2011. **106**(5): p. 407-11.
184. Gibson, P.G., et al., *Migration to a western country increases asthma symptoms but not eosinophilic airway inflammation.* Pediatric pulmonology, 2003. **36**(3): p. 209-15.
185. Zhang, K., et al., *Constitutive and inducible thymic stromal lymphopoietin expression in human airway smooth muscle cells: role in chronic obstructive pulmonary disease.* American journal of physiology. Lung cellular and molecular physiology, 2007. **293**(2): p. L375-82.
186. Neill, D.R., et al., *Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity.* Nature, 2010. **464**(7293): p. 1367-70.
187. Barlow, J.L., et al., *Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity.* The Journal of allergy and clinical immunology, 2011.
188. Saenz, S.A., et al., *IL25 elicits a multipotent progenitor cell population that promotes T(H)2 cytokine responses.* Nature, 2010. **464**(7293): p. 1362-6.
189. Barletta, K.E., K. Ley, and B. Mehrad, *Regulation of neutrophil function by adenosine.* Arterioscler Thromb Vasc Biol, 2012. **32**(4): p. 856-64.
190. Hasko, G. and P. Pacher, *Regulation of macrophage function by adenosine.* Arterioscler Thromb Vasc Biol, 2012. **32**(4): p. 865-9.
191. Rutgers, S.R., et al., *Airway inflammation and hyperresponsiveness to adenosine 5'-monophosphate in chronic obstructive pulmonary disease.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2000. **30**(5): p. 657-62.
192. Blackburn, M.R., et al., *Metabolic consequences of adenosine deaminase deficiency in mice are associated with defects in alveogenesis, pulmonary inflammation, and airway obstruction.* The Journal of experimental medicine, 2000. **192**(2): p. 159-70.
193. Cekic, C., et al., *Extracellular adenosine regulates naive T cell development and peripheral maintenance.* The Journal of experimental medicine, 2013.
194. Alessandrini, F., et al., *Effects of ultrafine carbon particle inhalation on allergic inflammation of the lung.* The Journal of allergy and clinical immunology, 2006. **117**(4): p. 824-30.
195. Alessandrini, F., et al., *Effects of ultrafine particles-induced oxidative stress on Clara cells in allergic lung inflammation.* Particle and fibre toxicology, 2010. **7**: p. 11.
196. Peternel, R., S. Music Milanovic, and L. Srnec, *Airborne ragweed (Ambrosia artemisiifolia L.) pollen content in the city of Zagreb and implications on pollen allergy.* Annals of agricultural and environmental medicine : AAEM, 2008. **15**(1): p. 125-30.

Publications

Pollen-derived adenosine is a necessary co-factor for ragweed sensitization

Maria Kamml, MSc[a,i]*, Francesca Alessandrini, PhD[a,i]*, Stefanie Gilles, PhD[a,i] , Ulrike Frank, PhD[b,i], Sebastian Oeder, PhD[a,i], Michael Hauser, PhD[c,i], Johannes Ring, MD[d,i], Fatima Ferreira[c], PhD, Dieter Ernst, PhD[b], Jana Barbro Winkler, PhD[e], Jenny Westphal, Dipl.Ing[f], Philippe Schmitt-Kopplin, PhD[f,g], Caspar Ohnmacht, PhD[a], Heidrun Behrendt, MD[a,i], Carsten Schmidt-Weber, PhD[a], Claudia Traidl-Hoffmann, MD[a,d,i], Jan Gutermuth, MD[a,d,h]

[a]Center of Allergy and Environment (ZAUM), Technische Universität and Helmholtz Center Munich, Member of the German Center for Lung research (DZL)
[b]Institute of Biochemical Plant Pathology/Helmholtz Zentrum München
[c]Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology University of Salzburg
[d]Department of Dermatology and Allergy Biederstein, TU Munich
[e]Research Unit Environmental Simulation at the Institute of Biochemical Plant Pathology, Helmholtz Zentrum München
[f]Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München
[g]Analytical Food Chemistry, Technische Universität München
[h]Department of Dermatology, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel, Brussels
[i]Christine Kühne – Center for Allergy Research and Education
Manuscript in preparation

Pollen extracts specifically enhance Th2-induced IgE production in B cells

Sebastian Oeder[1,6], Maria Kamml[1,6], Andrea Braun[5], Francesca Alessandrini[1,6], Ulrike Kanter[3,6], Michael Hauser[4,6], Joerg Durner,[3], Johannes Ring[2], Fatima Ferreira[4], Dieter Ernst[3], Martin Mempel[5], Carsten Schmidt-Weber[1], Heidrun Behrendt[1,6], Jan Gutermuth[1,2,7]

[1]Center of Allergy and Environment (ZAUM), Technische Universität and Helmholtz Center Munich
[2]Department of Dermatology and Allergy Biederstein, TU Munich
[3]Institute of Biochemical Plant Pathology/Helmholtz Center Munich
[4]Department of Molecular Biology, University of Salzburg
[5]Department of Dermatology, Venereology and Allergology, University of Göttingen
[6]Christine Kühne – Center for Allergy Research and Education
[7]Department of Dermatology, Vrije Universiteit Brussel, Brussels
Manuscript in preparation

B Lymphocytes as Targets of Environmental Pollutants: Gateway to Chronicity?

Sebastian Oeder[1,2], Maria Kamml[1,2], Jeroen T.M. Buters[1,2], Carsten Schmidt-Weber[1], Mübecel Akdis[2,3], Cezmi A. Akdis[2,3], Jan Gutermuth[1,2,4], Prof. Heidrun Behrendt[1,2]

[1] ZAUM – Center of Allergy and Environment, TU/ Helmholtz Center Munich
[2]Christine Kühne – Center for Allergy Research and Education (CK-CARE), Davos
[3]Swiss Institute of Allergy and Asthma Research (SIAF), University Zurich, Davos, Switzerland
[4]Department of Dermatology, Vrije Universiteit Brussel, Brussels
Manuscript in preparation

Presentations

Intranasal ragweed pollen extract induces initially a neutral innate response that is only followed later on by allergic inflammation
Oral presentation at the "25. Mainzer Allergie-Workshop 2013", March 7-8, 2013, Mainz, Germany

Rapid sensitization and exacerbation of preexisting bystander-inflammation by intranasal exposure with ragweed pollen extract
Poster presentation at the "EAACI Congress 2012", June 19-20, Geneva, Switzerland

Rapid induction of systemic Th2-responses by intranasal instillation of ragweed pollen extract
Oral presentation at the "24. Mainzer Allergie-Workshop 2012", March 22-23, 2012, Mainz, Germany

Rapid induction of systemic Th2-responses by intranasal instillation of ragweed pollen extract
Oral presentation at the "ADF-Jahrestagung", March 1-3, 2012, Marburg, Germany

A murine model to study the impact of climate change on the allergenic potential of ragweed
Poster presentation at the "6. Deutscher Allergie Kongress", September 8-11, 2011, Wiesbaden, Germany

Establishment of an *in vivo* model to analyze the allergenicity of ragweed pollen
Oral presentation at the "P3AGI Summer School: Animal Models of allergic Diseases", June 21-23, 2011, Vienna, Austria

Influence of climate change on the allergenicity of ragweed pollen: Results from environmental simulation chamber experiments
Oral presentation at the "23. Mainzer Allergie-Workshop 2011", March 10-11, 2011, Mainz, Germany

Influence of climate change on the allergenicity of ragweed pollen
Oral presentation at the "2. Treffen der AG Experimentelle Allergologie (AGEA)", March 17-19, 2011, Tübingen, Germany

Influence of sexual hormones on immunoglobulin production in B cells
Poster presentation at the "22. Mainzer Allergie-Workshop 2010", March 11-12, 2010, Mainz, Germany

Declaration

With this I declare that the presented work ,title **"Analysis of the sensitizing properties of ragweed pollen components and the influence of environmental factors in a murine *in vivo* model"** was written by myself and that I did not use other resources than those indicated. I did not undertake an unsuccessful graduation attempt and this thesis was not submitted to another institution before.

Acknowledgement

I am grateful for all the support I received from so many people during the last years.

First of all, I owe my gratitude to Prof. Heidrun Behrendt for giving me the opportunity to perform my doctoral research at the Center for Allergy and Environment.

I wish to thank my supervisor Dr. Jan Gutermuth for the continuous support of my Ph.D. research, for his patients, motivation, enthusiasm and the immense knowledge. Without his guidance and persistent help, this dissertation could not have been possible.

I would like to thank my thesis committee member and head of our institute Prof. Carsten Schmidt-Weber and my thesis committee member Prof. Jörg Durner for their encouragement, insightful comments and stimulating discussions.

In addition I am indebted to my colleagues who supported me during my work. In particular I would like to thank Francesca Alessandrini for all the help with the experiments, the analyses, and for always being there for me and answering to all my questions. To Johanna Grosch, Alexandra Seisenberger, Benjamin Schnautz and Katja Haslauer for their excellent technical assistance. To Tanja Seher and Viviana Marzaioli for the stimulating discussions and help and to all past and present group members and colleagues for all the fun we have had in the last years.

Last but not least, I owe my deepest gratitude to my boyfriend Christian, to my parents and my whole family, and to my friends for their unconditional love, their tremendous support and all the encouraging words during the years.