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The impact of climate change and pollution on the
allergenicity of *Ambrosia artemisiifolia* pollen

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1 Introduction

Ambrosia artemisiifolia is a highly invasive plant with allergenic pollen that causes rhinoconjunctivitis, asthma and atopic dermatitis. The rates at which it is spreading in Europe and the rates at which individuals are becoming allergic to it are on the rise. Studies indicate that air pollutants like diesel particles have adjuvant effects and that allergenic pollen responds to environmental changes such as extreme temperatures, changes in humidity and low pH by altering specific pollen surface enzyme expression. To determine whether these changes correlate with increased allergenicity, we investigated the effect on allergenicity of *Ambrosia* pollen using a mouse model of allergic asthma.

1.1 Aim of the study

1. Generate a suitable lab model for studying *Ambrosia artemisiifolia* pollen allergenicity.
2. Use the model to investigate the effect of environmental factors, such as climate change and environmental pollution, on the allergenicity of *Ambrosia* pollen.

These studies allow a better understanding of the rise in allergic disease, providing new insights into the pathology of pollen-induced asthma. As such, we generated a mouse model of pollen allergy that mimics human disease. Moreover, after developing the protocol for disease induction in BALB/c mice, we further used the model to investigate possible differences in allergenicity of pollen incubated under several stress conditions. Furthermore, we used the same mouse model to compare disease severity when induced with pollen from two distinct plant populations. The first sample population was harvested from a rural area outside Vienna city; unexposed to airway pollution (rural). For the second sample population we used plants collected from the vicinity of a highly motorized roadway within Vienna (urban) where plants are constantly exposed to exhaust fumes from the adjacent traffic.

1.2 Allergic asthma

Allergic asthma is a chronic immune-mediated inflammatory disease of the respiratory tract characterized by airway dysfunction induced by a hypersensitivity reaction against allergens. Both genetic and environmental factors contribute to the development of allergic airway diseases. In westernized societies allergic asthma is a major health problem as it continues to rise in incidence and prevalence since the last decades [1-3]. Moreover, a recent study

indicates that although the global burden of asthma is increasing, the global differences in prevalence have reduced. In addition, asthma symptom prevalence has reduced in westernized countries, but increased in Africa, Latin America and parts of Asia [4]. This could be correlated with the increase in communities adopting western lifestyle and becoming urbanized. It has been estimated that this changing lifestyle may lead to a further increase in the number of asthmatics worldwide over the next decades, leading to an additional 100 million people suffering of asthma by 2025 [5]. According to the World Health Organization (WHO), asthma is already affecting 235 million people worldwide and is the most common chronic disease among children [6]. People affected by allergic disease suffer from a series of clinical symptoms such as recurrent episodes of wheezing, shortness of breath, chest tightness, and coughing, particularly at night or in the early morning. Chronic inflammation of the airways is associated with hypersecretion of mucus, bronchia hyperresponsiveness, airway obstruction, and the inflamed tissue's increased susceptibility to environmental threats [7]. Allergic asthma is often under-diagnosed and under-treated, which can become a lifelong substantial burden to individuals and their families. Severe asthmatic manifestations can also lead to asthma related deaths, which are occurring mostly in the low and lower-middle income countries [6]. Besides the patient specific impairment, asthma is also associated with significant costs to society. Direct costs, such as hospitalization and medications, but also indirect costs, such as work and school loss, contribute to enormous healthcare expenditures, which are primarily correlated with comorbidities, age and severity of disease [8, 9]. In 2007 the Australasian Society of Clinical Immunology and Allergy calculated that the cost of allergy to the Australian economy is more than \$7 billion per year. Lower productivity, direct medical costs, lower employment rate, absenteeism, and lost household productivity together with premature death arise to this tremendous amount, of which the patients themselves bear most of the costs [10]. Another study conducted in 2003 in California estimated that the cost of asthma is \$4912 per person per year, and a 5% shift from severe to moderate asthma would save \$1,4 billion annually in the USA alone [9]. In 2001 The European Lung Foundation calculated that the total annual cost of care for asthma in the European Union amounts to €17,7 billion, of which €9,8 billion is related to lost workdays [11]. These examples give an insight in the huge economic burden of allergic asthma, underlining the serious threat allergic asthma represents by affecting health and economy worldwide.

1.3 Allergens

A wide range of allergens can trigger allergic asthma. The World Health Organization and International Union of Immunological Society Allergen Nomenclature Subcommittee defined an allergen as a protein or antigen that causes a specific IgE antibody response in at least 5 individuals [12]. Most of the known allergens are in the molecular size range of 10000 to 40000 Daltons and, although they do not have any distinguishing chemical features, they all induce a specific immune response in sensitive individuals [13, 14]. Allergens derive from various sources including pollen, dust mites, fungi, animal epithelia, insects, venoms, bacteria, foods, and drugs, and most of these allergenic sources do contain more than one allergen. Therefore, allergens are named by using the 3 letters of the genus, a single letter for the species and an Arabic number to indicate the chronology of allergen purification. Hitherto more than 1000 protein allergens have been sequenced and the number is steadily increasing [12, 15, 16]. In pollen of common grasses (Pooideae), eleven groups of allergens have been described, which are expressed as proteins or glycoproteins with distinct physicochemical properties. [14] Allergens are generally classified as major or minor allergens depending on their sensitization properties. Major allergens are defined by IgE recognition from more than 50% of allergic patients, by eliciting high specific IgE titers in this allergic population in association with clinical symptoms [14, 17]. Minor allergens are associated with less than 20% of the allergenic potential of grass pollen. The reactivity, recognition of patients and intensity of IgE response to these allergens, varies: Some are not frequently recognized by allergic patients but elicit a strong IgE response, while others are targets of a frequent, but mild IgE response [14]. The steadily increasing knowledge about the molecular and functional characteristics of allergens has improved classifications according to taxonomy and protein families. Furthermore, the standardization and characterization of allergen extracts has been improved, and novel hypoallergenic mutants have been developed for safe allergen-specific immunotherapy [18, 19]. Nevertheless, despite the great progress in molecular and clinical allergy research, what exactly makes some proteins clinically relevant in eliciting an immune response has not yet been determined. The question what makes an allergen an allergen remains unanswered [15].

1.4 *Ambrosia artemisiifolia*

Ambrosia artemisiifolia or common ragweed is an annual plant from the Asteraceae family. Being a native plant of North America, it is considered an invasive species in Europe and

other regions around the world. In the last decades, there was a massive spread of Ambrosia in several European countries, jeopardizing agricultural output and health. The main culpability for the Ambrosia invasion is attributed to the contamination of imported seed and feed with Ambrosia. Expansion of the road network with consequent increased traffic further assisted spread and proliferation, as vehicles are able to transport and distribute propagules. Mechanized harvesting used for cropping and mowing may have also contributed to the increased spread of Ambrosia in the last decades [20].

Ambrosia spread and growth is favored by bare and freshly mowed ground, which includes hot, dry, and rich soils with neutral or slightly acid pH. As a ruderal species it establishes populations preferably along roads, abandoned agriculture lands, gardens, and other environments disturbed by human activities, such as construction sites. It is a monoecious plant, producing both male and female flowers on the same plant. Every single plant can produce up to 60000 seeds, which are still capable to germinate after 40 years in the ground. Ambrosia plants usually attain a height of 20-120 cm, depending on environment and potential anthropogenic interventions [21].

The pollination period extends in central Europe usually from mid-July until end of September depending on seasonal variations [22]. Ambrosia plants produce large amounts of pollen: A single plant is able to produce up to 10^9 pollen grains. The pollen is between 18-22 μm in size with small non-sharp spikes on its surface and highly allergenic. A low concentration of 5-10 pollen grains per cubic meter can elicit allergic symptoms in susceptible subjects [20, 21]. Whole pollen grains are considered to be too big to reach the lower airways. Instead, sub-pollen particles (SPPs) measuring less than 5 μm are being released upon hydration, inducing an asthma attack. This also occurs during thunderstorms, when pollen particles burst, releasing sub-pollen particles. [23, 24].

Ambrosia pollen contain the major allergen Amb a 1, which elicits allergic reactions in more than 90% of Ambrosia-allergic people, and several minor allergens, such as Amb a 3, Amb a 4, etc. [20]. Ambrosia allergens are highly aggressive and the sensitization rate in the European population has been increasing for the last decades. In heavily infested regions, up to 12% of the population suffers from allergies to Ambrosia pollen. A study in Italy showed an increase of Ambrosia sensitization in allergic patients from around 25% in 1989 up to 70% in 2008. 90% of these patients suffer from allergic symptoms and 40% developed asthma [25]. In Austria, it has been shown that after a sensitization period of about 5 years, 30% of the exposed population develops allergic symptoms with 30% of the Ambrosia allergic patients progressing to chronic asthma [20].

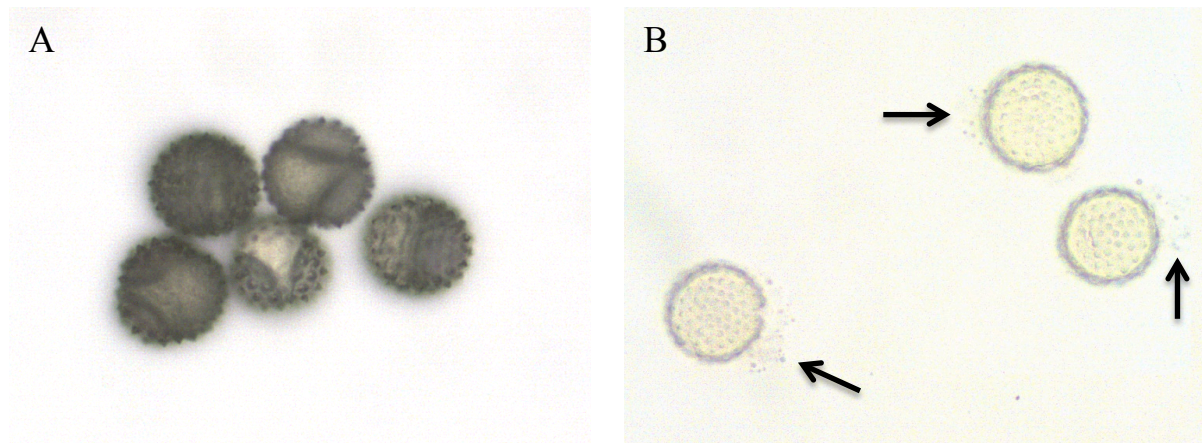


Figure 1: Photomicrographs of Ambrosia pollen: A: Ambrosia pollen in dry conditions. B: Ambrosia pollen upon hydration with release of sub-pollen particles. Black arrows indicate released sub-pollen particles.

The high allergenicity of its pollen and its quick spread throughout Europe and other regions around the world have made Ambrosia one of the most discussed research subject associated with the global rise of allergic asthma.

1.5 Pathogenesis of allergic asthma

Allergic inflammation is characterized by a complex interplay between several mediators, cell types, and pathways. Inflammatory cells, such as mast cells, eosinophils, basophils, lymphocytes, dendritic cells, and neutrophils, produce multiple inflammatory mediators including cytokines, chemokines, lipids, purines, and reactive oxygen species. They elicit their effects on target cells such as epithelial cells, fibroblasts, vascular cells, and airway smooth muscle cells that, in turn, produce further inflammatory response promoting symptoms. Moreover, the inflammatory gene expression is regulated by several transcription factors and epigenetic mechanisms, while defective endogenous anti-inflammatory mechanisms might also contribute to inflammation [26].

Asthma' chronic airways inflammation, characterized by airway wall remodeling and airway dysfunction, induces airway susceptibility to a wide range of environmental insults, including allergens and air pollutants etc. The result is an alteration of the repair response in airways with secretion of growth factors that induce mucus cell metaplasia, smooth muscle proliferation, angiogenesis, fibrosis and nerve proliferation [27].

1.5.1 Sensitization

Sensitization is the first step in the process that will eventually lead to allergic asthma. As a consequence to damage and activation of the respiratory epithelium, immature antigen-presenting cells (APCs) are induced by chemoattractants to migrate from the bone marrow to the epithelium and the underlying mucosa. These attracted cells mature to fully competent antigen presenting cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) [27]. Mature antigen-presenting cells, such as dendritic cells and macrophages, capture foreign antigens in the airway epithelium or mucosa. Sub-mucosal antigen-presenting cells are able to capture antigens that perforate through the mucosal epithelium either by means of their protease activity or in the case of an already disrupted epithelium. The antigen-presenting cells bind the antigens via the non-specific non-self receptors of the innate immune system and process them into small peptides. Activated antigen-presenting cells migrate to regional lymph nodes and present the processed antigen epitopes to naïve T cells. Once there, processed epitopes are presented via the major histocompatibility complex (MHC) class II and co-stimulatory molecules to the T-cell receptor of naïve $CD4^+$ T^H cells. The simultaneous presence of interleukin 4 (IL-4) and interleukin 13 (IL-13) induces maturation and differentiation of the naïve T cells to activated T^H2 effector and memory cells [27, 28]. Activated T^H2 cells interact with epitope-presenting B-cells, in the presence of T^H2 cytokines, to induce maturation of B-cells into plasma cells, followed by antigen-specific IgE production. Binding of the ϵ heavy chains of IgE to the high affinity IgE receptor Fc ϵ RI on mast cells and basophils completes the sensitization to the allergen [28].

1.5.2 Allergic response

Re-exposure to the sensitized allergen leads to release of mediators from mast cells and subsequent pathologic manifestations. Mast cell activation is induced by crosslinking of IgE binding antigen and Fc ϵ RI receptor complexes and results in a consequential release and new synthesis of mediators that cause the hypersensitivity reaction. Vasoactive amines, such as histamine, and lipid mediators, such as prostaglandin D_2 (PGD $_2$), leukotrienes, and platelet-activating factor (PAF), are the main responsible mediators for the immediate hypersensitivity reaction. The immediate hypersensitivity reaction is denoted by rapid vascular and smooth muscle reactions, such as vascular permeability, vasodilation, bronchoconstriction, and increased mucus production, occurring within minutes of antigen exposure [18, 27, 29]. Cytokines, such as tumor necrosis factor (TNF), Interleukin 4 (IL-4), Interleukin 13 (IL-13),

and Interleukin 5 (IL-5), contribute to the allergic reaction and mediate the late phase reaction. Activated mast cells and basophils together with activated T_H2 cells produce cytokines that recruit inflammatory leukocytes, including eosinophils, neutrophils, basophils, and T_H2 cells. Infiltration of neutrophils and monocytes is mainly directed by TNF, while eosinophils are mainly activated and recruited by IL-5. Activated eosinophils produce and release toxic proteins, such as cationic granule proteins and eosinophil peroxidase, which induce damage to airway tissue, bronchoconstriction, lung remodeling and promote the inflammatory process together with inflammatory mediators released from other recruited cells [18, 29].

1.5.3 Allergic asthma

Allergic asthma results from repeated allergen exposure and consequent immediate hypersensitivity and chronic late-phase reactions to allergens. Allergic asthma is characterized by chronic bronchial eosinophilic inflammation, edema, smooth muscle hypertrophy and hyperreactivity to bronchoconstrictors, mucus overproduction and reversible airway obstruction [29]. The asthmatic epithelium barrier function is impaired due to the intermittent inflammation status of the airways, thus facilitating the penetration of potentially harmful aeroallergens. IgE binds to the high-affinity $Fc\epsilon RI$ on mast cells and basophils to start the inflammatory cascade after antigen recognition. Furthermore, IgE is able to bind to the low affinity $Fc\epsilon RII$ on B cells to enhance both allergen-specific B cell and T cell memory functions. The allergic inflammatory cascade in asthma is conducted by T_H2 -type cytokines, produced from allergen-activated mast cells and allergen specific T_H2 cells. T_H2 cell survival is regulated by IL-4 and B-cell isotype switching to IgE synthesis by IL-4 and IL-13. Moreover, differentiation and maturation of mast cells is controlled by IL-3, IL-9 and IL-13, and maturation and survival of eosinophils is regulated by IL-3, IL-5 and GM-CSF [27]. Mediators released from recruited mast cells, basophils and eosinophils, such as leukotrienes, platelet-activating factor, TNF, IL-4, IL-5, IL-13 etc., are primarily responsible for bronchial constriction and inflammation [29].

1.6 Mouse as an asthma model

Most of the disease processes that are involved in asthma are not fully understood. As some studies are not applicable in human asthmatics, animal models are a valid approach to understand crucial pathways and pathologic consequences of asthma. The mouse is one of the

most widely used laboratory animal models to study human disease, mainly because of the availability of many different transgenic strains and mouse-specific reagents [30, 31].

Mouse models have been used to investigate the physiological and immunological processes of asthma. As asthma is a very complex disease, mice do not replicate all morphological and functional features of the human disease. However, mouse asthma models develop many characteristic key features of clinical asthma, such as eosinophilic lung inflammation, elevated levels of IgE, airway remodeling, goblet cell hyperplasia and mucus hypersecretion. Contrary to human patients, mice do not develop chronic allergic asthma after repeated allergen exposure, chronic exposure to allergen rather results in suppression of disease [32]. Additionally, mice do not show persistent metacholine-induced airway hyperresponsiveness (AHR) and while IgE and mast cells mediate immediate and late-phase allergic reaction in humans, they are not required to develop disease in mice. Furthermore, there are differences in physiology, lung anatomy, and eosinophil function between human and mice. Despite these differences, mouse models have been shown to be valid models to replicate and investigate human disease [32, 33].

The main strain for investigating allergic asthma is BALB/c. BALB/c mice are genetically predisposed towards a T_H2 immunological response after sensitization and challenge with allergens. Compared to other strains, they develop a more intense eosinophilic lung inflammation, higher levels of allergen-specific IgE and increased metacholine-induced airway hyperresponsiveness. Nonetheless, other strains such as C57BL/6, are often used for allergic disease models [34].

Most of the allergic models require multiple systemic administration of an allergen. A wide range of different allergens and various administration routes, such as nebulization, intratracheal (i.t.), intraperitoneal (i.p.) or intranasal (i.n.) administration, are used to elicit allergic disease. Additionally, adjuvants such as aluminum hydroxide are often administered to promote the T_H2 response. Chronic asthma models imply repeated exposure to allergen over a longer period and this can lead to tolerance, which can be prevented by administration of low dosages of allergen [30].

Evaluation models of disease usually include examination of bronchoalveolar lavage fluid (BAL), interpretation of histochemical stained lung sections, detection of immunoglobulin levels in sera and measurement of metacholine-induced airway hyperresponsiveness. Total and differential cell count of BAL give information about the severity of inflammation and the proportional distribution of different cell types, such as eosinophils, macrophages, lymphocytes, and neutrophils. Infiltration of inflammatory cells in the lung can be assessed by

hematoxylin and eosin (H&E) stained lung sections, and periodic acid-Schiff (PAS) stained lung sections are used to determine mucus secretion. Antigen-specific immunoglobulins, such as IgE, IgG1 and IgG2a can be detected in sera and several mediators, such as IL-4, IL-5, IL-13 etc., in BAL fluid [33].

Taken together, allergic asthma mouse models are valuable tools to examine allergic asthma and, despite some limitations, mice mimic important features of human disease. Mouse models have been crucial to investigate disease progress and interactions of cells and inflammatory mediators [30].

1.7 Gene-environment interactions in allergic asthma

Allergic asthma is a complex disease that can be caused by multiple biologic mechanisms and is encompassed by different etiologic and pathophysiologic factors. Complex interactions between multiple genetic and environmental factors contribute to the development of disease. Family history of allergic asthma is one important risk factor. The risk of developing disease increases twofold if one parent is affected and around fourfold when both parents are affected [35]. In the last decades, several studies tried to identify asthma-related genes and more than 100 candidate genes have been identified so far [36]. Most of the genes have been identified by hypothesis-driven studies that tried to find a correlation between variants, such as single nucleotide polymorphisms (SNPs) in the main pathways that influence allergic inflammation and asthma [37]. The most extensively studied candidate genes and single nucleotide polymorphisms associated with asthma are IL13, IL4, CD14, ADRB2, TNF, HLA-DRB1, FCERIB, and IL4R, among others [36]. These asthma-related candidate genes act in multiple pathways and their scopes are utterly distributed in the allergic reaction. Some of these genes are involved in triggering the immune response (e.g. CD14, HLA-DRB1), others regulate the T_H2-cell differentiation (e.g. IL4, IL13, FCERIB, IL4R), while still others, expressed by epithelial cells, interface between innate and adaptive immunity. Some genes are associated with lung function, airway remodeling and disease severity (e.g. ADRB2, TNF) [37]. Although all of these genes have been positively associated with asthma, in a substantial number of studies they either failed to show any correlation with asthma, or revealed only small effects [36, 38, 39]. In complex diseases such as asthma, problems with consistency and replication are not uncommon and may reflect variations in study design, size or analysis [40-42]. Some genes were found to be relevant in some populations, while in other populations they could not be associated due to the distribution of different functional variants within the

same gene in different populations. Furthermore, in most of the studies gene environment interactions have been neglected [43, 44]. Asthma features a very heterogeneous phenotype and many factors contribute to development and progression of disease. By contrast, other studies have shown that the individual effect of the asthma-associated genes on disease risk may be quite small [36]. Allergic asthma is not only a genetic disorder, but is strongly influenced by environmental factors that differ among populations. Both genetic and environmental factors contribute to the risk of developing asthma [45]. As mentioned above, developed countries are afflicted by a higher prevalence of asthma and allergy which cannot be explained only by genetic differences among ethnic groups [4]. Several environmental factors such as air pollution, chemicals, microbial exposures, diet, moisture and temperature have been shown to overlay with predisposed genetic background and influence the susceptibility of individuals to asthma. However, little is known about how and under which circumstances environmental factors interact with an exposed individual's genetic background [46]. One approach to explain the variation in rates of allergy and asthma by environmental factors is the hygiene hypothesis. The hygiene hypothesis is still evolving and it was initially formulated to explain the reduced rates of allergy among children with older siblings. Increased exposure to microbes and their products in the critical early period of immune maturation may be protective against allergic episodes to common environmental antigens [35, 47]. In the last few decades the hygiene hypothesis was extended to explain the differences in prevalence of westernized or urban societies and less developed rural societies, suggesting that improved living standards and higher hygienic environments increase the risk to develop allergic disease. Later on, the dominant T_H1/T_H2 paradigm was integrated, but when it became clear that both T_H1 -mediated autoimmune disease and T_H2 -mediated allergic disorders are increasing in westernized societies the hygiene hypothesis needed a further update. The current concept states that immune-regulatory mechanisms are mostly activated when interactions between the innate immune system and the microbial environment occur in utero and/or in early life [48-51]. The hygiene hypothesis is supported by abundant epidemiology and helps to explain some epidemiological scenarios, but some epidemiological data exceptions exists [52]. The essence of the hypothesis, stating that the microbial environment interacts with the innate immune system and modulates its ability to mediate directions to adaptive immune response, remains a valid point. This core of the hypothesis needs to be further integrated in the still incomplete understanding of the role of genetic variation in shaping immune responses to environmental stimuli [51, 53]. Epigenetics could represent an important link between genes and environment. It is the study of stable and

heritable (or potentially heritable) changes in gene expression without altering the DNA sequence [54]. The primary epigenetic mechanisms include DNA methylation, histone modification and other forms of chromatin remodeling, which are able to regulate gene expression. Epigenetic markers allow genetically identical cells to be structurally and functionally heterogeneous [55]. Stable epigenetic alterations can be inherited, but they can also be genotype-specifically obtained or modified in response to development and the environment. During life, environmental exposure and consequently gene-environment interaction may alter epigenetic marks, affecting risk for allergic asthma and other atopic diseases [56]. Most likely, these epigenetic-conducted gene-environment interactions occur primarily during early years [43].

1.8 Climate change, environmental pollution and the impact on human health

It is widely accepted that human-induced global warming and pollution has an impact on the biosphere and the environment, and it is widely accepted that climate change and pollution factors do impact human and environmental health.

1.8.1 Climate change

The on-going global warming is substantially related to the increase of global atmospheric concentrations of critical greenhouse gases. The Intergovernmental Panel on Climate Change (IPPC) states that the increase of critical greenhouse gases, including carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O), is primarily due to human activities that are superimposed on on-going natural variations in the climate. The global increase of carbon dioxide concentration is mainly associated with fossil fuel use and land use change, whereas methane and nitrous oxide are primarily associated with agriculture [57]. The anthropologic effect on increases in emissions and atmospheric concentrations can be observed since 1750, becoming even more evident since the mid-20th century. Over the last 100 years the temperature has increased by 0,74°C (0,56°C – 0,92°C), and it is projected to increase by 1,1°C – 6,4°C in the next 100 years, depending on which global warming scenario is followed [57, 58]. The global atmospheric concentration of carbon dioxide has also heavily increased in the last centuries and especially decades. The National Oceanic and Atmospheric Administration (NOAA) and Earth System Research Laboratory (ESRL) of the U.S.

Department of Commerce are performing the longest record of direct CO₂-measurement in the atmosphere. The NOAA/ESRL Global Monitoring Division on Mount Loa, Hawaii, measured an increase from 315 parts per million (ppm) in 1958 to 394 parts per million in 2012 [59]. In the last 50 years, the concentration of atmospheric CO₂ has increased by 22% and it will further increase, reaching an estimated concentration between 500 ppm and 1100 ppm by the year 2100. The exact dimension of global climate changes is depending on contemporary and future anthropogenic strategies especially in land-use, resource utilization, and energy production. The pattern of concernment will vary regionally, depending on the exposure to the respective changes [57]. Consequences to the anthropogenic greenhouse concentrations encompass warming of the oceans, changes in arctic temperatures and ice, glaciers melting, and diminished snow cover in the Northern Hemisphere. Furthermore, it includes widespread changes of intensity, frequency and type of precipitation, and an increase of extreme weather events, such as heat waves, droughts, floods and hurricanes [57, 60]. Diverse set of physical and biological systems all over the world are already affected by recent regional changes in climate, e.g. increase of temperature [57, 61].

1.8.2 Environmental pollution

In the last century industrial and economic growth has been associated with a massive increase in emissions of pollutants. Industrialization and urbanization involves environmental degradation with collateral contamination of air, soil, and water [62].

Human induced water contamination can be of many different kinds and origins. Water can be contaminated with microbial or chemical pollutants, such as heavy metals. The primary sources of water pollution are burning of fossil fuels, mining, municipal wastes, sewage, pesticides and fertilizers. Heavy metal exposure may lead to a wide range of disorders and diseases and is affecting people worldwide by groundwater contamination and/or through the food chain. Furthermore, heavy metals, pesticides, and fertilizer can act as ecological toxins in aquatic and terrestrial ecosystems [63].

The bioavailability of heavy metals in soils plays an important role for human toxicology and agricultural productivity. Soil, being an essential compartment for recycling and redistribution of nutritive elements, is a reservoir and source of pollution [64]. Industrial and traffic emissions and direct input from agricultural activities are the most important sources for soil contamination, and metal contamination in urban and industrial areas is higher than in rural areas [65, 66]. Regarding human health, agriculture, and ecotoxicology, arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), technetium (Tc), and uranium are the most

concerning heavy metals. Other metals, such as cobalt (Co), chromium (Cr), copper (Cu), manganese (Mn), molybdenum (Mo), and zinc (Zn) are essential in small concentrations for plants and/or animals but can be toxic in high concentrations [64]. Copper, cadmium, and zinc soil contamination is mostly associated with agriculture activities, such as sewage sludge and fertilizer spreading [64, 66].

1.8.2.1 Copper

Copper is one of the essential heavy metals but it is also potentially toxic. Average concentrations range from 70 mg/kg in the lithosphere to around 30 mg/kg in soils, where copper forms sulphides, sulphates, sulphosalts, carbonates and other compounds. Under reducing environments it is also occurring as the native metal. Anthropogenic input in form of soil additions from smelter, fertilizer, sewage sludge and other waste, fungicides, and bactericides, is mostly responsible for elevated concentration levels of copper in the surface soil horizon [67]. In agriculture copper is widely used as a pesticide in form of copper hydroxide ($\text{Cu}(\text{OH})_2$) and copper oxychloride ($\text{Cu}_3\text{Cl}_2(\text{OH})_4$). In 1995 20% of global fungicides were copper solutions. Although restrictions of copper usage have been established in many countries because of its toxic characteristics, it is still widely used, being the only organic option to fight fungi [68].

1.8.2.2 Ammonium nitrate (NH_4NO_3)

Ammonium nitrate is another compound that is widely used in agriculture. It is one of the most commonly used nitrogen fertilizer worldwide, accounting for 43% of the total used fertilizer in Europe, and it is also a significant component of the atmospheric aerosols [69, 70]. In many ecosystems, nitrogen supply is a key factor in controlling the nature and diversity of plants and animals. Excessive ammonium nitrate use can lead to pollution near bodies of water with severe consequences for aquatic wildlife [71]. As a nitrogen fertilizer, ammonium nitrate is a source of greenhouse gases and contributes to both global warming and eutrophication. Ammonium nitrate's contribution to particulate matter (PM) fluctuates and is reported to be higher during spring and summer in Sweden [72], while it is higher during winter in Switzerland [73].

During the last century economic and industrial growth has generated a massive increase in emissions of air pollutants. Air quality has become one of the major environmental problems

worldwide, being an issue especially in urban and industrial areas. The most abundant air pollutants are nitrogen dioxide (NO₂), ozone (O₃), and particulate matter (PM) in urban areas, and sulphur dioxide (SO₂) in industrial areas [74].

1.8.2.3 Nitrogen dioxide

Nitrogen dioxide is an oxidant pollutant that is generated mainly by combustion of fossil-derived fuels and, therefore, primarily found in urban and industrial regions. It is a precursor of photochemical smog and, in conjunction with sunlight and hydrocarbons, it results in the production of ozone [60]. The decline of coal burning and the change to gas or nuclear power decreased nitrogen dioxide emissions in the last decades in Europe, except in some Southern European countries [75]. Nitrogen dioxide exposure may increase prevalence of asthma and rhinitis in asthmatic subjects [60].

1.8.2.4 Ozone

Ozone is a powerful oxidant and may account for up to 90% of total oxidant levels in urban and industrial areas. It is generated at ground level by photochemical reactions involving nitrogen dioxide, hydrocarbons, and ultraviolet radiation. Elevated atmospheric concentrations of ozone are associated with an increased rate of respiratory diseases [60].

1.8.2.5 Particulate matter

Particulate matter is a major component of urban air pollution, consisting of a mixture of organic and inorganic solid and liquid particles of different origins, sizes, and composition. Contributing mechanisms to and composition of particulate matter are very variable and are region-specific. Traffic, industrial and agriculture aerosols are the main sources contributing to particulate matter composition. Inhalable particles are classified according to their aerodynamic diameter: PM₁₀ is smaller than 10 µm, PM_{2.5} is smaller than 2.5 µm and PM₁ has an aerodynamic diameter smaller than 1 µm [73]. Common major element sources are nitrate; mineral and organic matter, such as ammonium nitrate, ammonium sulphate; and exhaust emissions of traffic containing a high region-variable diversity of trace elements, such as copper and other metals [73, 76]. Inhalation of particulate matter is associated with an enhanced rate of several respiratory diseases, including asthma, and represents a worldwide severe health threat [77].

1.8.2.6 Sulphur dioxide and acidification of water and soil

Sulphur dioxide is an air pollutant that is mainly generated by combustion of sulphur containing coal and fossil fuel, and inhalation of sulphur dioxide is associated with several obstructive effects in the respiratory tract [60]. Sulphur acid is the most important substance that leads to acidification of water and soils, and both, nitric acid (HNO_3) and sulphur dioxide, are the two main components that lead to acid rain. Acid rain can scavenge and incorporate atmospheric gases and aerosol particles [78]. Acid rain involves terrestrial and aquatic acidification, which affects vegetation and fauna. Damages to natural ecosystems, such as loss of fish populations and decreased agriculture yields, have been common consequences of acidification [79]. Since 1990, emissions of sulphur acid decreased in several European countries by up to 80%. Because both emissions of sulphur dioxide and nitric acid are primarily generated by anthropogenic sources, the reduction of both emissions has led to a decrease of acidified water and soil [80].

Due to the massive increase in air emissions during the last century, new pollution policies were set up which led to an improvement in the quality of air in the last decades. Substantial emission reduction was achieved by cost-effective provisions proposed by scientific organs, such as the Convention on Long-Range Transboundary Air Pollution (CLRTAP) of the United Nations Economic Commission for Europe (UNECE) and has led to a decrease in deposition of eutrophying and acidifying compounds [80].

Emission category	Actual emissions		Emission reductions relative to 1990 (%)
	1990	2010	
SO_2	37,0	7,9	$\cong 79$
NO_x	23,7	12,8	$\cong 46$
NH_3	7,6	4,9	$\cong 36$
$\text{PM}_{2,5}$	6,6	2,7	$\cong 59$

Table 1: European pollutant emissions. Emissions from several European countries are displayed in million tons from 1990 and 2010 and in percent reductions relative to emissions from 1990. Data used from Reis et al. [80].

The recent reduction in emissions of pollutants is a step in the right direction, but while some pollutant emissions are decreasing in some regions of the world, in other regions pollutant emissions are still increasing. Besides, current pollution levels of air, water, and soil are still high enough to be responsible for several threats to human and environmental health.

1.9 Climate, pollution and asthma

The impact of climate change and pollution on asthma is still not clarified and represents a hot topic of investigation. It has been established that climate patterns and different pollution factors may have an impact on the initiation and/or exacerbation of allergic reactions. Climate and pollution can directly impact the airways of patients, making them more susceptible to asthma, or indirectly by influencing the physiology and distribution of living organisms, such as plants and fungi. Temperatures and greenhouse gases affect the timing and quantity of allergenic aeroallergen production, and pollution factors may interact directly with allergens, increasing allergenicity. Additionally, climate changes affect the different aerobiological processes such as emission, dispersion, transport, and deposition of aeroallergens [74, 81].

1.9.1 Aeroallergen production

In the last decades we witnessed an increase in atmospheric pollen, which may be related to climate changes. The impact of climate change on regional weather patterns and atmospheric CO₂ concentrations can lead to enhanced pollen production and consequently to higher pollen concentrations. The shift in aeroallergen production may result in an increased risk of sensitization and exacerbation of asthma and may also lead to more severe allergic reactions [82]. Several studies have shown that aeroallergen production increases when plants are growing in habitats with elevated CO₂ concentrations and/or temperatures. Increased temperature seems not only to impact on the pollen-production of plants, but tends also to prolong the pollen-production period. A study in Switzerland could relate increased airborne pollen quantities from hazel, birch, and grass to climate change. While the increased annual mean temperature induced hazel to flower earlier, there was only a slight shift for grass and no significant shift of the flowering period for birch. In addition the hazel and grass pollen count correlated positively with air temperature. By contrast the birch pollen count did not correlate with temperature, but correlated negatively with precipitation [83]. Another study, examining the responses of western ragweed (*Ambrosia psilostachya*) to experimental warmed soil, displayed an increase of 105% in aboveground biomass and 84% in total pollen production compared to normal control plots. The increase of total pollen production was not triggered by an increase of pollen production per stem, but due to increased number of stems. Furthermore, pollen from plants that were grown in warmed soil had an increase of 13% in pollen diameter compared to controls [84].

CO₂ enrichment can also have a strong impact on pollen producing plants. Increased CO₂ concentrations can influence the biomass, form, and physiology of plants. A meta-analysis of elevated CO₂ effects on woody plants showed a total biomass and CO₂ assimilation increase when plants were affected by twice-ambient CO₂ concentration. CO₂ stimulated total biomass response was further increased by low light, while it was reduced by low soil nutrient availability [85]. Another meta-analysis on plant reproduction under elevated CO₂ conditions showed a strong CO₂ mediated increase of biomass. Comparing data of 79 crop and wild species, CO₂ enrichment (500-800 ppm) leads to increased flower (+19%), fruit (+18%), and seed (+16%) production. Furthermore, CO₂ enrichment resulted in greater individual seed mass (+4%), greater total seed mass (+25%), and lower seed nitrogen concentration (-14%). They also found differences among individual taxa and among functional groups. Compared to undomesticated species, crop species, growing in a CO₂ enriched environment, allocated more mass to reproduction and produced more fruits and seeds [86]. The CO₂ mediated effect on biomass, as for instance the increase of flower production, implicates a higher pollen production. Several studies confirmed that CO₂ enriched environment increases pollen production of allergenic species. One indoor study found significantly increased pollen production when common ragweed (*Ambrosia artemisiifolia*) plants were grown at elevated (700ppm) CO₂ concentrations, compared to plants grown at ambient (380 ppm) CO₂ concentration [87]. Another study comparing the responses of common ragweed to elevated CO₂ concentration detected changes in biomass and pollen production. Ragweed plants grown in elevated CO₂ environments (700 ppm) showed higher biomass production and an increase of 61% in pollen production, compared to plants grown in ambient CO₂ environment (350 ppm). The average size of pollen grains was not affected significantly by CO₂ [88]. An in situ investigation compared pollen production of ragweed plants growing in rural areas versus plants growing in urban areas. Consistent with most global climate scenarios, CO₂ concentration and air temperature within the urban environment were around 30% and 1,9 °C higher than within the rural site. Urbanly located plants grew faster, flowered earlier, and produced significantly more aboveground biomass and pollen than rurally located plants. Besides the CO₂ and temperature induced increase in pollen production, this study implicates that urbanization, which shows similar climate patterns than future projected climate change, might already be accountable for public health consequences [89].

Climate change might also have an impact on mold growth. Climate change and moisture are important predictors of mold spore counts, but the effect varies by mold species and geography [90]. It has been shown that elevated CO₂ concentrations (500 and 600 ppm)

induced *Alternaria alternata*, a ubiquitous fungus, to produce more spores than lower concentrations (300 and 400 ppm) [91].

Taken together, this array of studies indicates that climate factors have a significant impact on aeroallergen production. Shifting the pollen production period may vary and/or prolong the detrimental season for sensitive people to encounter aeroallergens. An increased aeroallergen production may lead to a higher atmospheric concentration and subsequently to more frequent and/or more severe episodes of asthma.

Besides shifting and increasing the amount of aeroallergen production, climate change may also have an impact on spatial distribution of the allergenic plants and pollen. Global warming is shifting the geographic habitat of several plant species farther north, which may change global and regional pollen distribution, and consequently, expose people to new and/or more allergens [92, 93].

1.9.2 Direct impact of climate and pollution on humans

The human lung is constantly exposed to a large quantity of different particles. Under usual circumstances the particles are repelled by the lung's defensive mechanisms, such as mechanical removal and biochemical neutralization. Exposure to toxic particles or to an excess of particles may lead to damage of the mucosal membrane in the lungs, facilitating the penetration of aeroallergens and their interaction with cells of the immune system. Consequently, small amounts of particles may be able to induce harmful effects in lungs, impaired by disease, toxic particles and/or an excess of particles [94].

Several studies have correlated climate and air pollution with increased susceptibility to respiratory diseases and increased mortality [95-97]. It is suggested that air pollution may facilitate allergic sensitization in susceptible individuals, affect pulmonary development in children, and reduce lung function [98-100]. NO₂, sulphur dioxide, ozone, and particulate matter are the most discussed pollutants that are linked to increased exacerbation of asthma. The specific effects depend on the type of pollutant, its environmental concentration, and the duration of exposure [96, 101].

New evidence suggests that exposure to nitrogen dioxide, although not definitively correlated with notable changes in bronchial function, may lead to enhanced allergic response or reduce lung function in asthmatic subjects [101, 102].

Inhalation of sulphur dioxide can induce an immediate bronchoconstriction in asthmatic subjects and enhance susceptibility to allergens. Impairment of lung function can already occur 2 minutes after exposure to SO₂, whereas repeated exposure to SO₂ may lead to

tolerance. There is clear evidence that exposure to SO₂ restrains lung function and enhances asthma susceptibility, but the mechanisms have yet to be completely clarified [60].

Exposure to elevated ozone concentration can lead to impairment of lung function, enhanced susceptibility to allergens, and to an increased risk of asthma exacerbation and mortality. It is proposed that ozone directly affects the airways by an increased production of intracellular reactive oxygen species and an increased permeability of epithelial cells that facilitates inhaled allergens to penetrate the airways. Furthermore, ozone exposure increases airway inflammation and airway responsiveness [103, 104].

Exposure to particulate matter has been associated with an increased prevalence and aggravation of asthma and has been correlated with higher mortality. It has been observed that inhalation of particulate matter may increase susceptibility to asthma in susceptible individuals, reduces lung function, and increases symptoms in asthmatic subjects. Fine particulate matter (<PM_{2.5}) may be able to penetrate deep into airways and induce alveolar inflammation [60, 98, 105]. It is suggested that transition metals may be mainly responsible for the adverse health effect of particulate matter. Metals, such as iron, chromium, cobalt, and copper, among others, may generate hydroxyl radicals and other reactive oxygen species that damage the airways [94, 106, 107].

Particles from diesel exhaust particulate (DEP) are able to reach the lower airways and cause headache, fatigue, nausea, cough, sputum production, and impairment of lung function. Inhalation of diesel exhaust particulate may modify the immune response in predisposed subjects and enhance susceptibility to allergens. Diesel exhaust particulate is able to bind proteins and may act as a carrier for allergens [108, 109]. Polycyclic aromatic hydrocarbons (PAH) may be the key agents causing the harmful effects of diesel and gasoline exhausts. These similar chemical compounds bearing fused aromatic rings, are primarily generated by incomplete combustion of fossil fuel. Inhaled particles are targeting bronchial epithelial cells and induce cytokine expression initiating a pro-inflammatory response. These molecules are able to interact with DNA and modify growth and differentiation programs of cells [110, 111]. Intranasal challenge with diesel exhaust showed to change the nasal cytokine expression. After challenge with diesel exhaust particulate, nasal cells had increased mRNA levels of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and interferon- γ (IFN- γ) [112]. Consecutive studies have investigated the effect of combined diesel exhaust particulate and Ambrosia allergen Amb a 1 in humans with Ambrosia allergy. In contrast to intranasal challenge with ragweed alone, combined of diesel exhaust particulate and Amb a 1 challenge resulted in an enhanced T_H2-type cytokine expression. Expression of mRNA for IL-4, IL-5, IL-6, IL-10 and

IL-13 were elevated. Furthermore, combined challenge increased Ambrosia-specific IgE compared to challenge with Ambrosia alone. IgE isotype switching could be observed after combined challenge, but not after challenge with diesel exhaust particulate or Amb a 1 alone. These studies conclude that the combination of mucosal stimulation with diesel exhaust particulate and Ambrosia allergen is able to induce isotype switching to IgE in susceptible subjects and represents a key feature in increasing allergen-induced respiratory allergic diseases [110, 113].

Copper is a transition metal that enhances generation of reactive oxygen species. Similar to diesel exhaust particulate, it has been shown that copper is able to damage the mucosal membrane in the airways and induce a pro-inflammatory response. Copper-induced oxidative damage leads to macrophage activation with consequent release of pro-inflammatory mediators, such as IL-6, IL-8, TNF α and NF- κ B, which further amplify the inflammatory response [94, 114].

1.9.3 Impact of climate and pollution on allergens

Climate and pollution may also have a direct impact on aeroallergen composition and structure. Meteorological variables, such as temperature, humidity, and pollution were shown to be able to change viability, germination, enzyme expression, and allergen-concentrations of pollen. There is evidence that these and other climate and pollutant-related factors may have an influence on pollen allergenicity.

Exposure of pollen (*Malus domestica* and *Corylus avellana*) to stressful environments, such as increase in temperature and humidity, copper and acid rain pollution showed negative impact on viability and germination, and changes in enzymatic expression. Acid rain (pH 5,6), CuCl₂ pollution (3,1 μ g/L), increased temperature and humidity (30°C, 100% relative humidity) caused, primarily in *Corylus* pollen, an increase in externalized transglutaminase activity, compared to natural conditions. The researcher propose that the stress-induced extracellular pollen transglutaminase might be a mediator of pollen allergenicity [115].

Another study analyzes the impact of ozone on pollen (*Ambrosia artemisiifolia*) viability, reactive oxygen species and nitric oxide (NO) content, activity of nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase, and expression of major allergens. Ozone fumigation reduced pollen viability, while reactive oxygen species and nitric oxide contents were not affected. While ozone did not change the expression of the Amb a 1 allergen, reactive oxygen species-generating enzyme NAD(P)H oxidase was significantly increased.

The researcher suggest that ozone may increase Ambrosia allergenicity by stimulation of NAD(P)H oxidase [116].

Several studies indicate that climate and pollution are able to affect pollen allergenicity by altering the content of allergen in pollen. Birch (*Betula pubescens*) pollen from trees growing in a warmer environment, compared to pollen from an environment with lower daily mean temperatures, elicited a higher IgE-response in sera of birch allergic patients. These results indicate that temperature is able to affect the amount of allergen in pollen [117].

Ryegrass (*Lolium perenne*) pollen from plants that were affected by elevated ozone concentrations showed an increase in group 5 allergen compared to pollen that was exposed to lower ozone concentrations. The study showed elevated expression patterns of Lol p 5 in ryegrass after elevated ambient ozone levels in three experimental series [118].

Comparing the Amb a 1 content of Ambrosia pollen under different CO₂ concentrations has brought evidence that CO₂ is able to impact on specific allergen content of pollen. Although total pollen protein was not affected, pollen from plants exposed to projected future CO₂ conditions (600ppm), compared to pollen from plants exposed to pre-industrial (280 ppm) and current CO₂ conditions (370 ppm), showed a significant increase in Ambrosia major allergen Amb a 1 [119]. Another study examined the impact of traffic and pollution on the allergenicity of Ambrosia pollen. Pollen collected from urban plants exposed to high traffic elicited a stronger allergen-IgE reactivity in pooled sera of Ambrosia-allergic patients than pollen from rural areas. Quantitative as well as qualitative differences in allergen patterns were detected. Pollen from high-traffic areas contained more total allergenic protein and more Amb a 1, but also a wider spectrum of single allergens, such as Amb a 6 and Amb a 10 [120]. Nitration of pollen may also contribute to allergic disease, as nitrated pollen was shown to exhibit an increased potency to trigger immune responses. It has been indicated that nitration of Bet v 1 allergen from birch (*Betula verrucosa*) pollen enhances antigen processing and presentation by increasing both the quality and quantity of the Bet v 1-specific peptide repertoire [121].

These outcomes indicate that climate and pollution-induced modifications of pollen may increase the pollen allergenicity and that there might be a species-specific impact.

Climate and pollution may also impact on the pollen structure, modifying the cell wall structure and facilitating the release of sub-pollen particles (SPPs). Sub-pollen particles are small pollen particles that are released by a biochemically active process upon hydration. Sub-pollen particles are able to reach the lower airways and to induce reactive oxygen species

generation and inflammation as they contain NAD(P)H oxidases, Amb a 1, and other antigenic compounds [23].

Pollen from blue grass (*Poa annua*), when grown in cadmium contaminated soil, showed an increased release of sub-pollen particles compared to pollen from plants that were grown in uncontaminated soil. Cadmium-exposed pollen released higher amounts of allergenic proteins and the release onset was earlier. Furthermore, more single grass pollen allergens were found in pollen from cadmium-affected grass [122]. No detectable differences of sub-pollen particle release were found in a study comparing *Ambrosia* pollen from high-traffic pollution areas and low-traffic rural areas. The researcher suggest that differences in the cell wall structure, which is slicker and thicker in *Ambrosia*, might be a possible explanation for these findings [120].

Besides long-period climate changes, extreme weather events and thunderstorms may also contribute to allergic asthma. Pollen may be disrupted or hydrated during thunderstorms and consequently release allergic sub-pollen particles. This suggestion would partly explain the linkage of asthma epidemics to thunderstorms [24].

Together, these findings suggest that there is an association between climate change, air pollution, and asthma. Many investigations imply a possible impact of environmental factors, but so far there are not many *in vivo* studies performed to support these indications. Nevertheless, both climate and pollution seem to contribute to development and exacerbation of asthma and aeroallergen production, allergen concentrations and aeroallergen allergenicity can be affected by a various spectrum of factors. Although there are some discrepancies and contradictory studies, there is plenty of evidence that climate and pollution-induced modifications might play an important role in the emergence of allergies.

2 Material and methods

2.1 Animals

Four to eight week-old female BALB/c mice from Charles River (Germany) were used in all experiments. Animals were housed in a specific pathogen-free facility at the Medical University of Vienna. Mice were maintained in a temperature-controlled environment with a strict 12-hour light-dark cycle with free access to food and water. All experiments complied with the requirements of the Animal Care Committee of the University of Vienna Medical School and were preapproved by the Austrian Ministry of Science.

2.2 Pollen

Ambrosia artemisiifolia pollen was purchased from Allergon, Sweden (90% purity; 25% total protein content). The pollen was stored at 4°C.

2.2.1 Pollen collection sites

To compare the allergenicity of pollen derived from urban and rural Ambrosia plant populations, pollen was collected from locally harvested plants (*Figure 2*). Three different sampling sites in and around Vienna were chosen in collaboration with Ao. Univ. Prof. Dr. Gerhard Karrer from the University of Natural Resources and Life Sciences, Vienna (BOKU). First population (rural) plants were taken from an abandoned rural field outside the city and away from any populated area. Plants growing here were not affected by any potential air pollution, either industrial or highway traffic. Plants from the other two populations (urban) were harvested alongside a highly circulated highway within 10-50 meters from the pavement. Plants here were subjected to intense pollution with exhaust fumes, as well as soil contamination resulting from highway traffic and road maintenance.

Rural pollen: Sampling site 1 was located in a rural area near Leobendorf in Niederösterreich (+48° 22' 52.17", +16° 18' 8.95"). We identified a large undisturbed Ambrosia population, consisting of tall full-grown plants in an uncultivated potato field (*Figure 3*), which did not seem to have been affected by mowing or any human interference. Plants were collected on 23.08.2012.

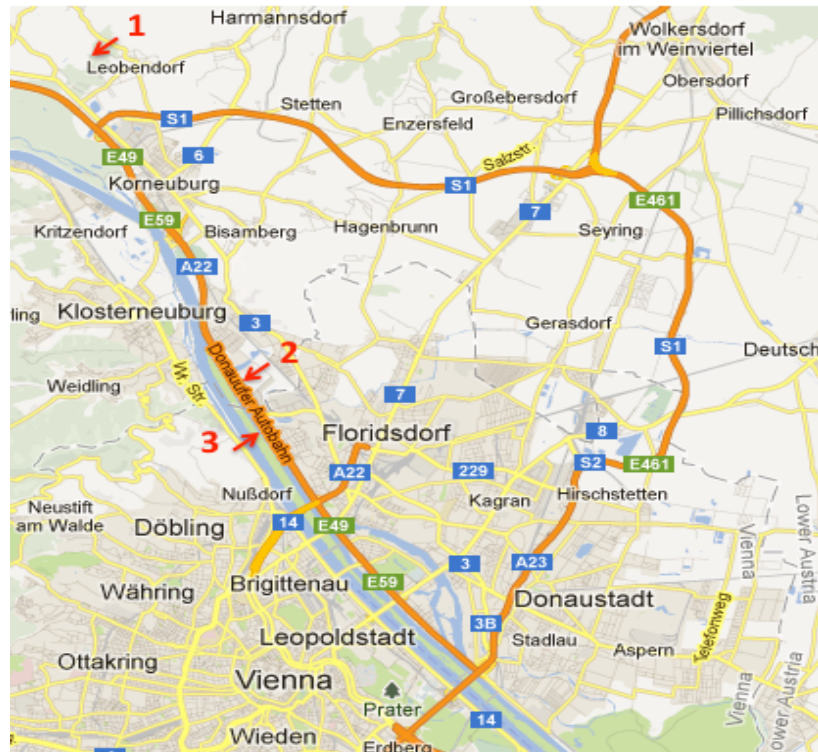


Figure 2: Map with the location for the sampling sites. Red arrows indicate the sampling areas: 1. Rural sampling site located near Leobendorf; 2. Urban sampling site I; 3. Urban sampling site II.



Figure 3: Sampling site of the rural Ambrosia plant population

Urban pollen I: Sampling site 2 was located near Vienna, on the eastern roadside of the Donauufer Autobahn, a main motorway with heavy traffic (+48° 17' 27.62", +16° 21' 25.16"). This Ambrosia plant population was growing in the close proximity of the motorway, where they were directly exposed to the traffic exhaust fumes. A small industrial zone in the close vicinity and urban pollution from Vienna may have further affected plants and pollen. The plants were small in size, due to regular mowing as part of roadside maintenance. Plants were harvested on 23.08.2012.

Urban pollen II: Sampling site 3 was located on the western roadside of the Donauufer Autobahn in the urban region of Vienna (+48° 16' 39.036" +16° 22' 3.54"). Ambrosia plants were located within 20-30 meters of the highway and exposed to the traffic exhaust pollution and urban pollution of Vienna. The site is characterized by fallow land and disturbed soil that has been dredged from the Danube and the Danube island. The Ambrosia population was widely spread with well-developed plants, some extending to 180cm. Medium size plants were collected. Plant harvest was on 07.09.2012.

2.2.2 Pollen harvest

60-90 flowering and pre-flowering Ambrosia plants per population were harvested and transferred to a glasshouse. Plants were transferred with their roots in water vases to assure viability and each population was stored in a separated compartment to prevent mixing of the pollen. Clean sheets of paper were laid out under each vase (*Figure 4*). Four to five days later, dropped pollen was carefully collected from the paper with small brushes. The yield varied among populations between 1 and 7 grams, depending most notably on size and viability of the collected plants. Using this approach, we were able to collect approximately 3 grams from plants coming from sampling site 1 (rural pollen), less than 1 gram from sampling site 2 (urban pollen I) and around 6 grams of pollen from sampling site 3 (urban pollen II).



Figure 4: Installation of harvested Ambrosia plants. Plants including their roots were kept in water vases and each population was stored in separated compartments of the glasshouse.

2.2.3 Pollen treatment

To investigate the impact of environmental factors on pollen allergenicity we subjected commercially purchased pollen to an increase in temperature and several pollution scenarios. Ambrosia pollen was treated with elevated temperature, low pH solutions, copper chloride (CuCl_2 , Sigma-Aldrich), and ammonium nitrate (NH_4NO_3 , Sigma-Aldrich).

2.2.3.1 Heat treatment

To mimic a heat wave ranging for two days, we incubated pollen at 40°C in an incubator for 48 hours.

2.2.3.2 Acid rain treatment

Acid rain was mimicked by incubating pollen in solutions with low pH values. To create the solutions for the acid rain treatment, we adjusted tap water with sulfuric acid (H_2SO_4) and nitric acid (HNO_3) at a ratio of 2:1. Next, 100mg of pollen was incubated for one hour in 4ml of each prepared solutions (pH2, pH3, pH4 and pH5.6). After incubation, the solution with

suspended pollen was transferred into an open Petri dish and left to dry overnight in a laminar hood. Once the water evaporated, the pollen was collected and stored at 4°C for further use.

2.2.3.3 *Copper pollution*

Pollen was suspended in different dilutions of distilled water and copper chloride. We prepared dilutions with a copper chloride final concentration of 3,1µg/L, 10µg/L, 100µg/L and 1000µg/L. We then incubated 100mg of pollen in 4ml of CuCl₂ solution for one hour, followed by transfer into a petri dish and drying overnight. Pollen was then collected and stored at 4°C for further use.

2.2.3.4 *Ammonium nitrate pollution*

We incubated pollen in solutions with different concentrations of ammonium nitrate to mimic ammonium nitrate pollution scenarios. Ammonium nitrate solutions were prepared at a final concentration of 10µg, 100µg and 1000µg per liter. After incubating 100mg of pollen in 4ml of each solution, we transferred it into petri dishes and left it to dry in a laminar hood. Pollen was then collected and stored at 4°C for further use.

2.3 Asthma model

To induce allergic asthma, mice were treated intra-nasally with repeated doses of ragweed pollen, for 6 times over 3 weeks. Prior to administration, pollen was suspended in 50 µl of phosphate buffered saline (PBS, DPBS Dulbecco's Phosphate Buffered saline, Gibco) and administered it intranasal (i.n.) to the mice. Before each pollen treatment, mice were anesthetized lightly via intra-peritoneal injection with a dose of approximately 50 mg/kg Ketanest (ketamine-hydrochloride; Pfizer) and 2 mg/kg Rompun (xylazin-hydrochloride; Bayer AG). Animals were challenged, on day 0, 2 and 4, 13, 15 and 17. 72 hours (day 20) after the last challenge mice were sacrificed and diseases was evaluated (*Figure 5*). Mice were killed with an intra-peritoneal injection dose of approximately 200 mg/kg Ketanest and 24 mg/kg Rompun. We then evaluated airway and lung inflammation, Ambrosia-specific antibody titers and mucus secretion. Mice treated with PBS only were used as control.

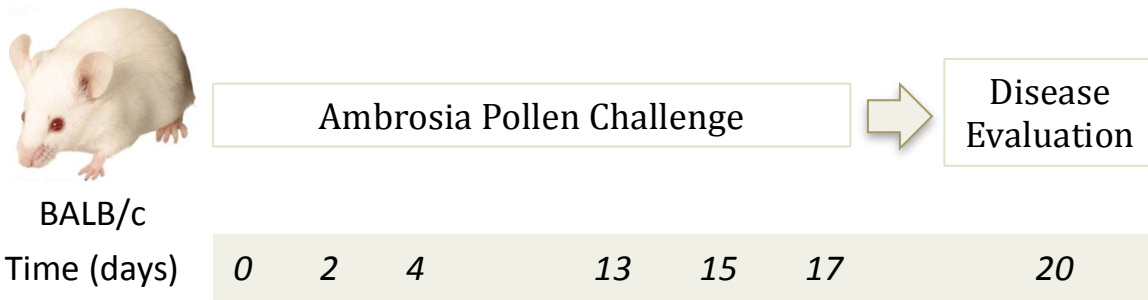


Figure 5: Scheme of the experimental protocol to induce allergic asthma in BALB/c mice.

2.3.1 Airway inflammation

72 hours after the last pollen challenge, mice were evaluated for inflammation and mucus hypersecretion. The tracheas of lethally anesthetized mice were cannulated and lavaged with 1 ml of PBS. Total leukocytes in the bronchoalveolar lavage fluid (BAL) were counted with a hemocytometer (modified Neubauer chamber) and a light microscope after Trypan Blue staining. Cytospin slides were stained with Diff-quick-staining (Thermo Fisher Scientific) to determine the cell differential count. Minimum 300 cells were counted per each sample

2.3.2 Lung inflammation and mucus hypersecretion

Following bronchoalveolar lavage, lungs were perfused with 1ml 4% Paraformaldehyde (PFA) and subsequently imbedded in paraffin. 3µm-thick sections of the paraffin-imbedded lungs were cut with a microtome (HM400, Microm, Heidelberg) and stained with hematoxylin and eosin (H&E) for morphological evaluation and with periodic acid-Schiff (PAS) for mucopolysaccharide staining, which reflects mucus production. Peribronchial inflammatory infiltrates and mucus hypersecretion were determined with a light microscope (Olympus BX41) and evaluated with a semiquantitative score (0-3).

We used the following scoring system to evaluate the extent of inflammation in the lungs: Grade 0: no inflammatory infiltrates; Grade 0,5: little inflammatory infiltrates in/around central airways; Grade 1: abundant inflammation in/around central airways; Grade 1,5: little inflammation in/around middle airways; Grade 2: abundant inflammatory infiltrates extending to middle airways; Grade 2,5: few inflammatory infiltrates in the periphery of the lungs; Grade 3: abundant inflammation in/around peripheral airways.

To evaluate the mucus production we used the following score: Grade 0: no mucus producing cells; Grade 0,5: few mucus producing cells in the central airways; Grade 1: abundant mucus production the central airways; Grade 1,5: few mucus producing cells in the middle airways;

Grade 2: abundant mucus producing cells in the middle airways; Grade 2,5: few mucus producing cells in the periphery airways; Grade 3: abundant mucus production in the peripheral airways.

Representative photomicrographs of H&E and PAS stained lung sections were taken with cell^B Image Acquisition Software (Olympus) and Olympus SC20 camera coupled to the light microscope.

2.3.3 Serum IG

Blood was collected through cardiac puncture (approximately 0,5 ml) to determine levels of circulating Ambrosia-specific immunoglobulins (IgE, IgG1 and IgG2a) by Ig-ELISA. Sera were collected after centrifugation of blood sample for 10 minutes at 15000rpm. 96-well ELISA-plates were coated with Ambrosia extract (10µg/ml PBS) and incubated overnight at 4°C. On the following day, the plates were washed with washing buffer, containing 0,1% Tween 20, (Bio-Rad) and blocked with 2% BSA for two hours at room temperature. After washing, serial dilutions of sera were added and incubated overnight at 4°C. Starting dilution was 1:5 for IgE and 1:500 for IgG1 and IgG2a (in 1% BSA/PBS) and subsequently diluted 1:10. Next, plates were washed and incubated with biotinylated anti-Ig (IgE: 1:250, BD Pharm 553419, IgG1: 1:1000, Southern Biotech 1070-08; IgG2a: 1:500, Southern Biotech 1080-08) for two hours at 4°C. Plates were washed again and incubated with Streptavidin-HRP (1:8000, Southern biotech, 7100-05) for 1 hour at room temperature. After washing, plates were incubated in darkness with TMB substrate (3,3', 5,5' tetramethylbenzidine, BD Biosciences) for 10min at room temperature. Finally stop solution was added and OD450 was measured with a fluorometer.

2.3.4 Statistics

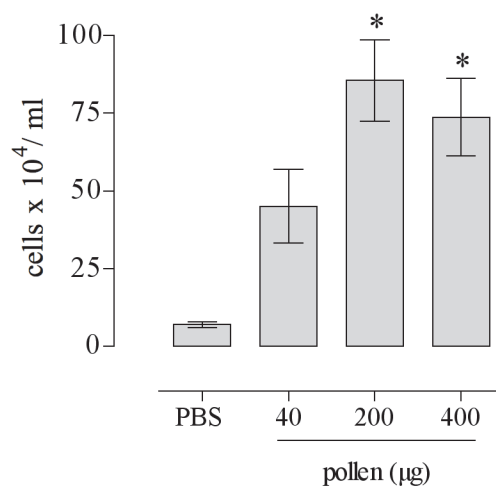
Data were analyzed by GraphPad Prism (Version 4.0a) program for Macintosh applying one-way ANOVA or t-test nonparametric measurements with following Bonferroni post-tests.

3 Results

3.1 Development of the allergic asthma protocol

Our first approach was to develop a protocol that induces allergic asthma to Ambrosia pollen in mice. Previous experience in our lab showed that six-time intranasal challenge with an allergen is able to induce allergic asthma in mice. As such, we challenged the mice six times intranasally with Ambrosia pollen, and assessed disease 72 hours after the last challenge. Pollen titration experiments were required to determine the optimal dose that will be further used in experiments. We then administered doses of 40, 200 and 400 μ g pollen, which corresponds to 10, 50 and 100 μ g total protein per each challenge. 72 hours after the last challenge, mice were sacrificed, followed by blood, bronchoalveolar lavage (BAL) and lung collection for downstream processing.

Total and differential cell counts were counted in BAL, which reflects the expected increase in inflammatory cells within the airways (Figures 6 & 7). Cell numbers were increased in all pollen-treated mice when compared to PBS-treated control, with significant differences for the 200 and 400 μ g groups (Figure 6). We observed the total cell numbers in BAL in PBS-treated mice to be at around 70000 cells/ml, while in pollen-treated animals the numbers were between 450000 cells/ml (40 μ g pollen) to 850000 cells/ml (200 μ g pollen), and around 470000 cells/ml (400 μ g pollen).



*Figure 6: Airway inflammation. Total cell count after repeated administration of Ambrosia pollen induced a significant increase of the total cell numbers in BAL when comparing to mice treated with PBS. Data are expressed as means $\pm$ SEM; $n=5$. *Significant to PBS group. Data were compared with one-way ANOVA and Bonferroni post-test, $p < 0,01$.*

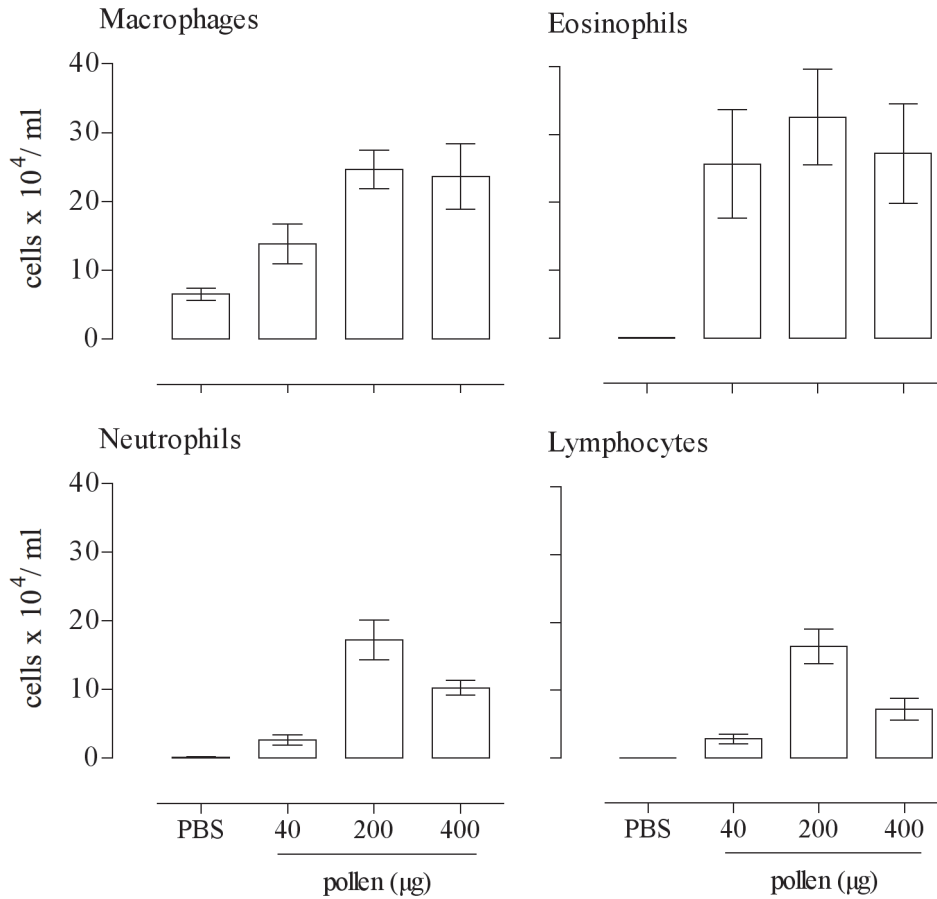


Figure 7: Inflammatory infiltrates in BAL of mice with acute disease following pollen challenge. Differential cell numbers for leukocytes per milliliter of BAL. Eosinophils are the most abundant cell type in BAL fluid of pollen-treated mice, followed by macrophages, neutrophils and lymphocytes. BAL fluid of PBS-treated mice consisted mainly of macrophages. Data are expressed as means $\pm$ SEM; n=5.

Differential cell count (Figure 7) following Diff-quick-staining of fixated BAL cells indicated eosinophils as the biggest fraction of BAL cells in pollen-treated animals, followed by macrophages. Neutrophils and Lymphocytes had little contribution to the BAL cell composition. In all three pollen-treated groups, eosinophils were strikingly increased and featured percentages between 31% and 54%. By contrast the percentage of eosinophils was less than 1% in the PBS-treated control group, where BAL fluid was constituted primarily of macrophages (around 99%).

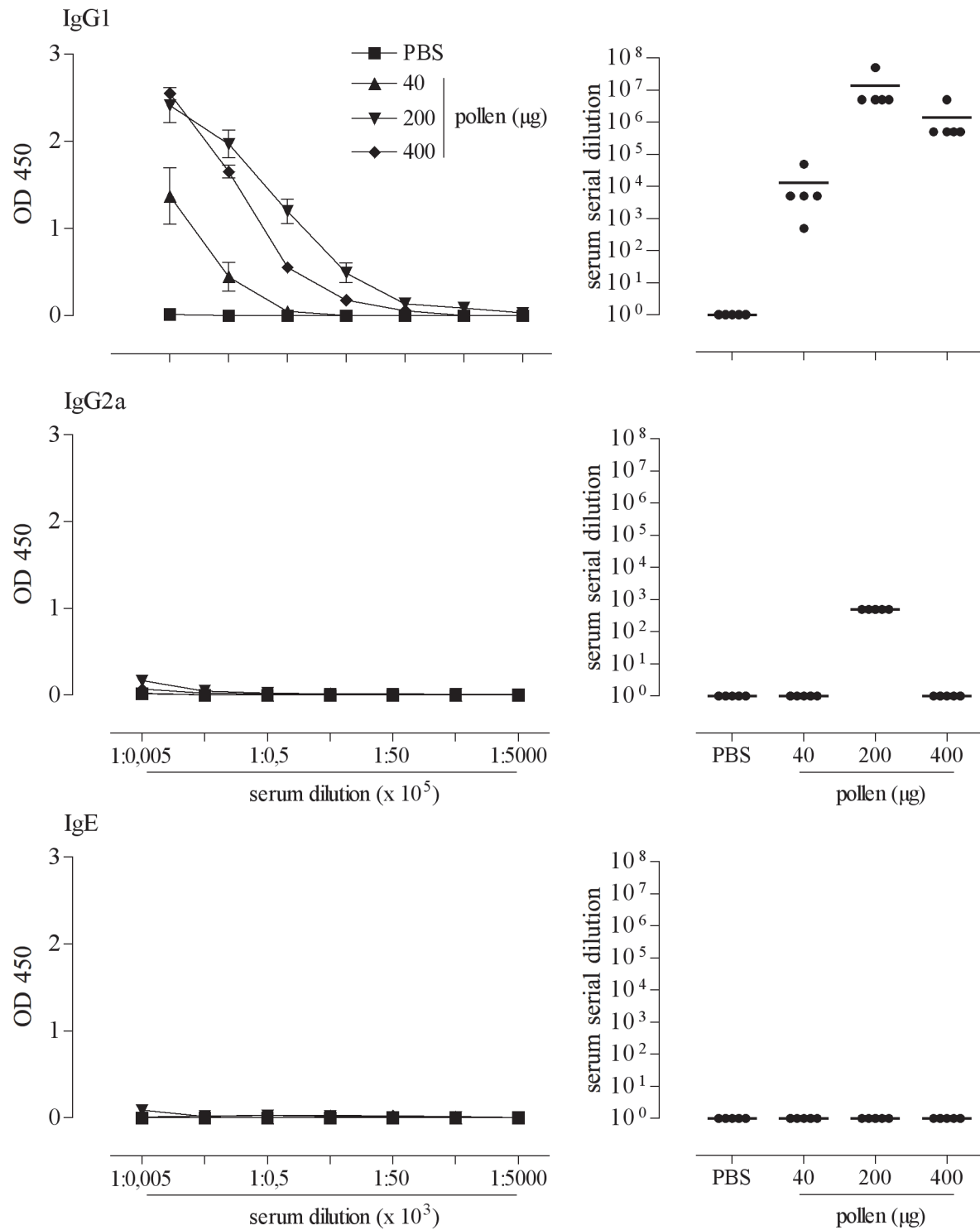


Figure 8: Immune response to pollen following *i.n.* administration of allergen. Serum antibody titers for *Ambrosia*-specific IgG1, IgG2a and IgE. Application of higher pollen doses (200 and 400 μg) resulted in a higher IgG1 titer compared to the 40 μg dose, and no signal was detected in PBS-treated mice. IgG2a and IgE were not detectable in pollen- or in PBS-treated groups. Immunoglobulin curves are shown on the left side and reciprocal titer levels on the right side. Data are expressed as mean OD450 nm $\pm$ SEM; $n = 5$.

Using ELISA, Ambrosia-specific antibodies levels in serum were evaluated (Figure 8). Intranasal application of Ambrosia pollen led to high Ambrosia-specific IgG1 titers. All three pollen-treated groups showed a significant increase in Ambrosia specific IgG1 levels compared to control PBS-treated group. Mice treated with 40 μ g pollen had a lower titer than mice treated with 200 and 400 μ g pollen. IgG2a levels were not detectable, except for a very low titer in the sera of mice treated with 50 μ g pollen. IgE signal was absent.

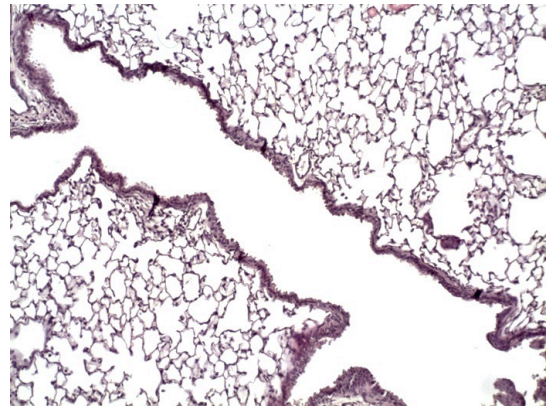
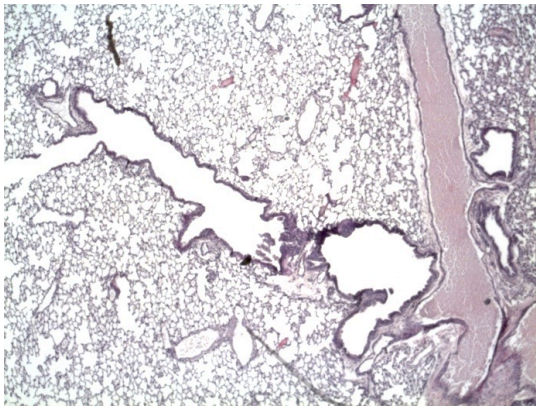
Next, lungs were processed and sections were stained with hematoxylin and eosin (H&E) to evaluate lung inflammation. Representative photomicrographs of H&E-stained lung sections of mice treated with PBS or 40 μ g pollen are shown in Figure 9. Lungs from pollen-treated mice had abundant lung inflammation around the airways with eosinophilic infiltration (Figure 10).

To evaluate lung inflammation we used a previously designed scoring system as follows: Grade 0 equals no inflammatory infiltrates; Grade 0,5: little inflammatory infiltrates in/around central airways only; Grade 1: abundant inflammation in/around central airways; Grade 1,5: central airways are inflamed with little inflammation in/around middle airways; Grade 2: abundant inflammatory infiltrates extending to middle airways; Grade 2,5: central and middle airways have inflammation with few inflammatory infiltrates in the periphery; Grade 3: abundant inflammation spreading from central all the way to peripheral airways. All three groups of pollen-treated mice showed a similar level of lung inflammation with inflammatory infiltrates spreading to the peripheral airways.

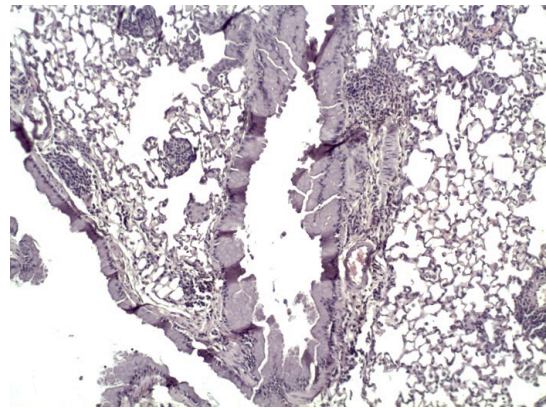
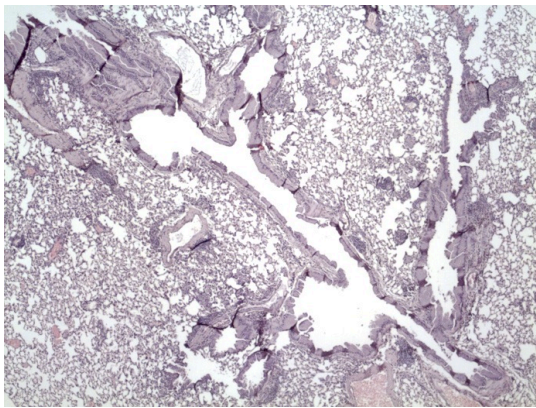
PBS

4X

10X



Pollen



Pollen 40X

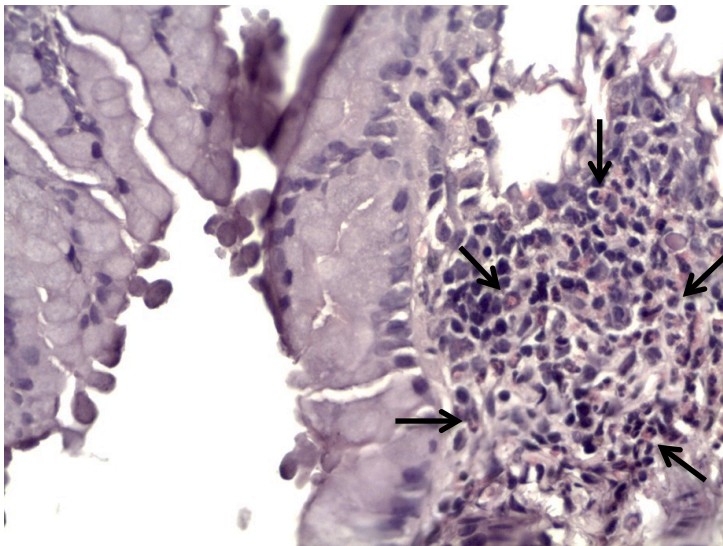
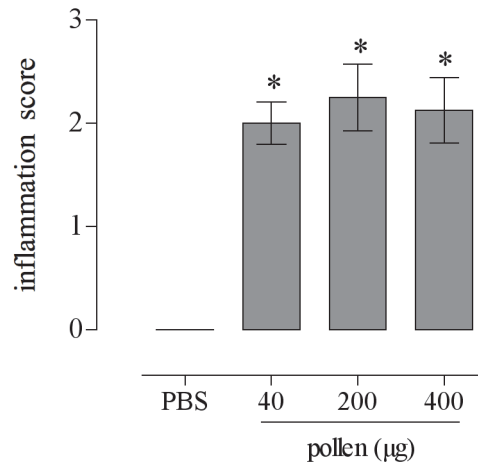


Figure 9: Eosinophilic inflammatory infiltrates in the lungs of mice with acute disease following intranasal challenge. Representative photomicrographs of H&E-stained lung sections comparing PBS- and pollen-treated mice at 4X, 10X and 40X objective lens respectively. High power field (40X) illustrate eosinophils in pollen-treated mice (black arrows) and persistent infiltrates containing lymphocytes, plasma cells and macrophages.



*Figure 10: Inflammation scores in H&E-stained lung sections after repeated administration of several Ambrosia pollen doses revealed as increased inflammation in all pollen-treated animals compared to PBS-treated mice. Inflammation score was significantly different in all pollen groups versus mice treated with PBS only. Grade 0 represents no inflammation, while an increased grade is associated with an increase in abundance and distribution of inflammatory infiltrates. Data are expressed as means $\pm$ SEM; $n=5$. *Significant to PBS group. Data were compared with one-way ANOVA and Bonferroni post-test, $p < 0,001$.*

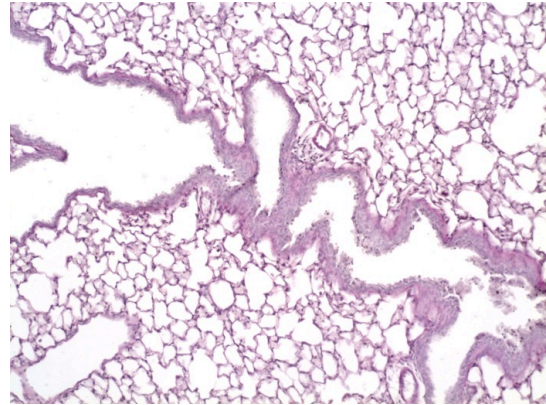
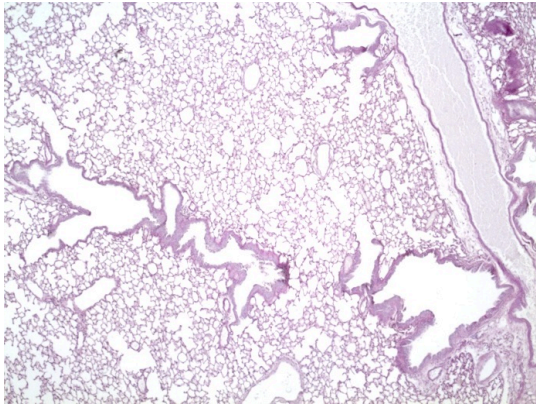
To evaluate mucus production of lung epithelial goblet cells, lung sections were stained with periodic acid-Schiff (PAS). Representative photomicrographs of PAS-stained lung sections of mice treated with PBS or 40µg pollen are shown in Figure 11. PAS-stained lung sections of pollen-treated revealed a high abundance and distribution of mucus producing epithelial lung cells, while mice treated with PBS did not show mucus production or only very few goblet cells strewn around the lungs (Figure 12). Similar levels of mucus distribution in central and middle airways with epithelial goblet cells reaching the peripheral airways observed in all pollen-treated groups.

To evaluate mucus production we used a previously designed scoring system as follows: Grade 0: no mucus producing cells; Grade 0,5: few mucus producing cells in the central airways; Grade 1: abundant mucus production the central airways; Grade 1,5: few mucus producing cells present in the middle airways; Grade 2: abundant mucus producing cells in the middle airways; Grade 2,5: few mucus producing cells in the peripheral airways; Grade 3: abundant mucus production in the peripheral airways. Significant differences comparing mice treated with PBS versus 200 and 400µg of pollen. Data are expressed as means $\pm$ SEM; $n=5$. Data were compared with one-way ANOVA and Bonferroni post-test, $p < 0,001$.

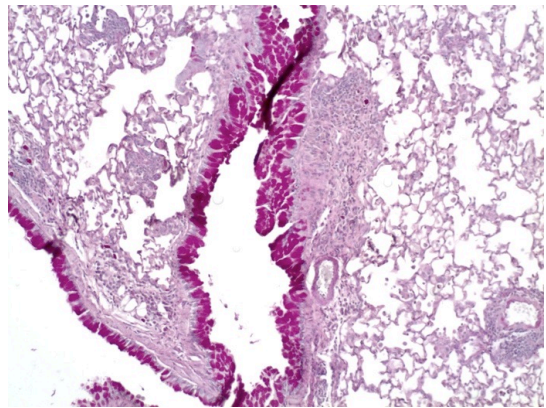
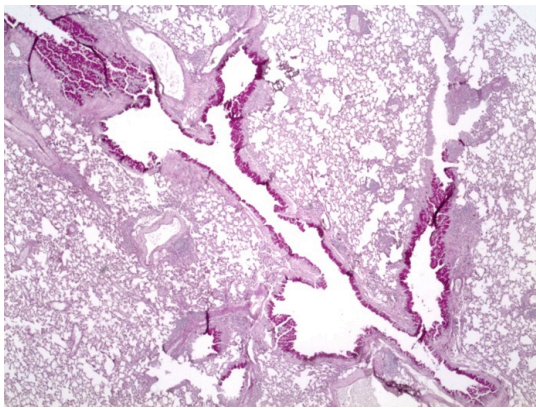
PBS

4X

10X



Pollen



Pollen 40X

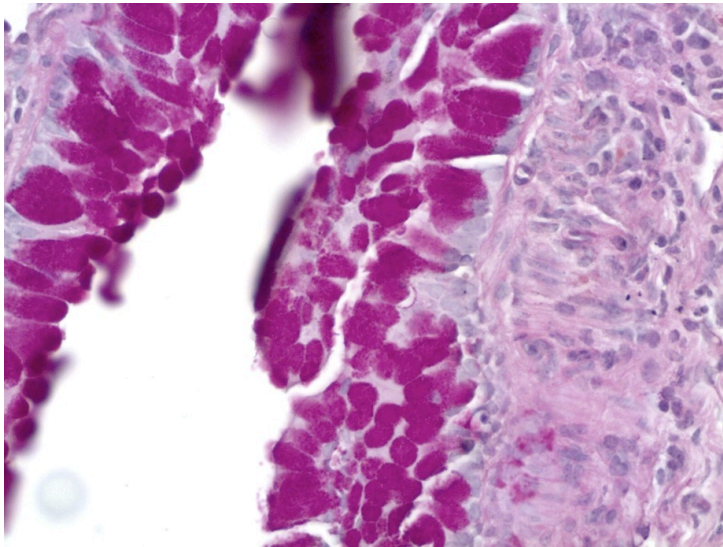
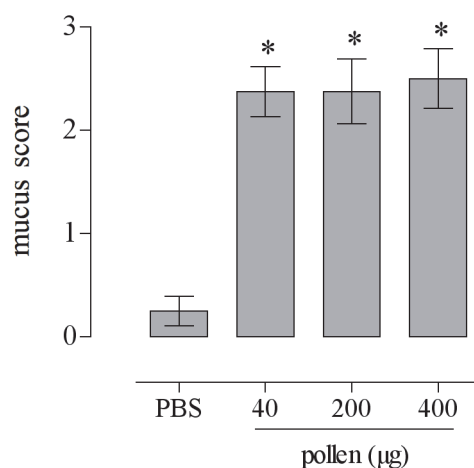


Figure 11: Mucus hypersecretion in mice with acute disease following intranasal pollen challenge. Representative photomicrographs of paraffin-embedded PAS-stained lungs at low (4X, 10X) and high (40X) power fields for pollen- and PBS-treated mice. Intense fuchsia staining of mucopolysaccharide within goblet cells of the respiratory epithelium and the airway lumen demonstrate mucus production.



*Figure 12: Mucus scores in PAS-stained lung sections after repeated administration of several Ambrosia pollen doses revealed mucus hypersecretion in all pollen-treated animals compared to PBS-treated mice. Mucus score was significantly different in all pollen groups versus mice treated with PBS only. Grade 0 represents no mucus production, while an increased grade is associated with an increase in abundance and distribution of mucus production. Data are expressed as means $\pm$ SEM; $n=5$. *Significant to PBS group. Data were compared with one-way ANOVA and Bonferroni post-test, $p < 0,001$.*

In conclusion, six-time administration of Ambrosia pollen in mice induced a significant increase of total inflammatory cell numbers in BAL fluid compared to mice treated with PBS. The composition of BAL fluid cells in pollen-treated mice was dominated by inflammatory eosinophils, followed by macrophages. In contrast, PBS-treated mice showed very low numbers of cells in BAL fluid that consist mainly of macrophages. While Ambrosia specific IgG1 titers were elevated in sera of pollen-treated mice, IgG1 was not detectable in PBS-treated mice. IgG2a and IgE titers were under the detection capability in both PBS- and pollen-treated groups. Furthermore, lungs of pollen-treated mice showed mucus hypersecretion and inflammation with infiltrated inflammatory cells.

3.2 Impact of environmental effects on pollen allergenicity

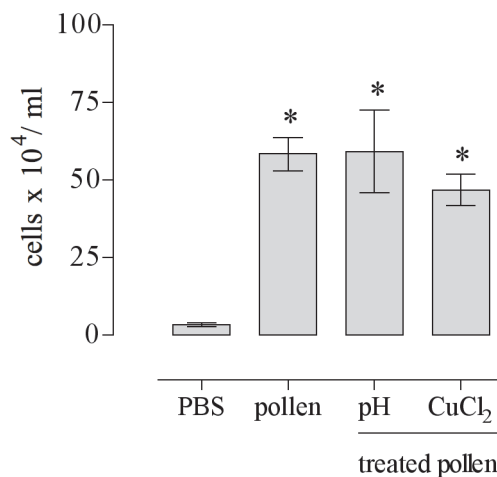
3.2.1 Treatment of pollen with different environmental factors to determine possible changes of pollen allergenicity

After successfully establishing a suitable model for studying ambrosia induced asthma in mice, we assessed the possible impact climate and air pollution may have on Ambrosia pollen allergenicity. To address that, we incubated Ambrosia pollen under several environmental

stress factors. We included heat, acid rain scenarios and copper chloride (CuCl_2) and ammonium nitrate (NH_4NO_3) pollution scenarios in our experiment.

In our first approach we mimicked scenarios of acid rain and CuCl_2 pollution. Pollen was incubated in solutions of pH5,6 (H_2SO_4 : HNO_3 at a ratio of 2:1) to simulate acid rain or CuCl_2 ($3,1\mu\text{g/L}$) to mimic airway and/or soil pollution. To assess possible allergenic differences, one group of mice was challenged with untreated pollen, while mice treated with PBS were used as negative control. A total pollen dose of $40\mu\text{g}$ pollen per challenge was used which corresponds to $10\mu\text{g}$ of protein.

Animals were sacrificed and disease assessed accordingly. For airway inflammation, BAL inflammatory cell count was determined as mentioned previously. Cell numbers were highly increased in all pollen-treated mice showing significant differences compared to PBS-treated control mice (Figure 13). Total cell numbers of BAL in PBS-treated mice were around 34000 cells/ml while in pollen-treated animals the numbers varied from around 580000 cells/ml (untreated pollen), to around 590000 cells/ml (pH-treated pollen) and around 470000 cells/ml (CuCl_2 -treated pollen). No significant differences between the pollen-treated groups were detected.



*Figure 13: Airway inflammation. Total cell count in BAL from pollen-treated groups and PBS control group comparing airway inflammation after application of treated (pH and CuCl_2) and untreated pollen. Significant differences comparing PBS-treated mice versus mice treated with pollen, but no significant differences in between different pollen treatment groups were observed. Data expressed as means $\pm$ SEM; $n=5$ *Significant to PBS group. One-way ANOVA and Bonferroni post-test, $p < 0,01$.*

Evaluation in the BAL (Figure 14) revealed that the composition of BAL cells in PBS-treated mice was dominated by macrophages (ca. 91%) with small fractions of Eosinophils (3%), Neutrophils (5%) and Lymphocytes (1%). On the other hand, in all three pollen-treated groups eosinophils represented the biggest fraction of BAL cells, ranging from 53% in the untreated pollen group and 52% in the pH-treated pollen group to 56% in the CuCl₂-treated pollen group. Macrophages represented between 33% and 36%, neutrophils between 5% and 10 %, while Lymphocytes ranged from 3% to 5% of total cells present. No significant differences in the composition of BAL cells could be found when comparing the three groups treated with pollen.

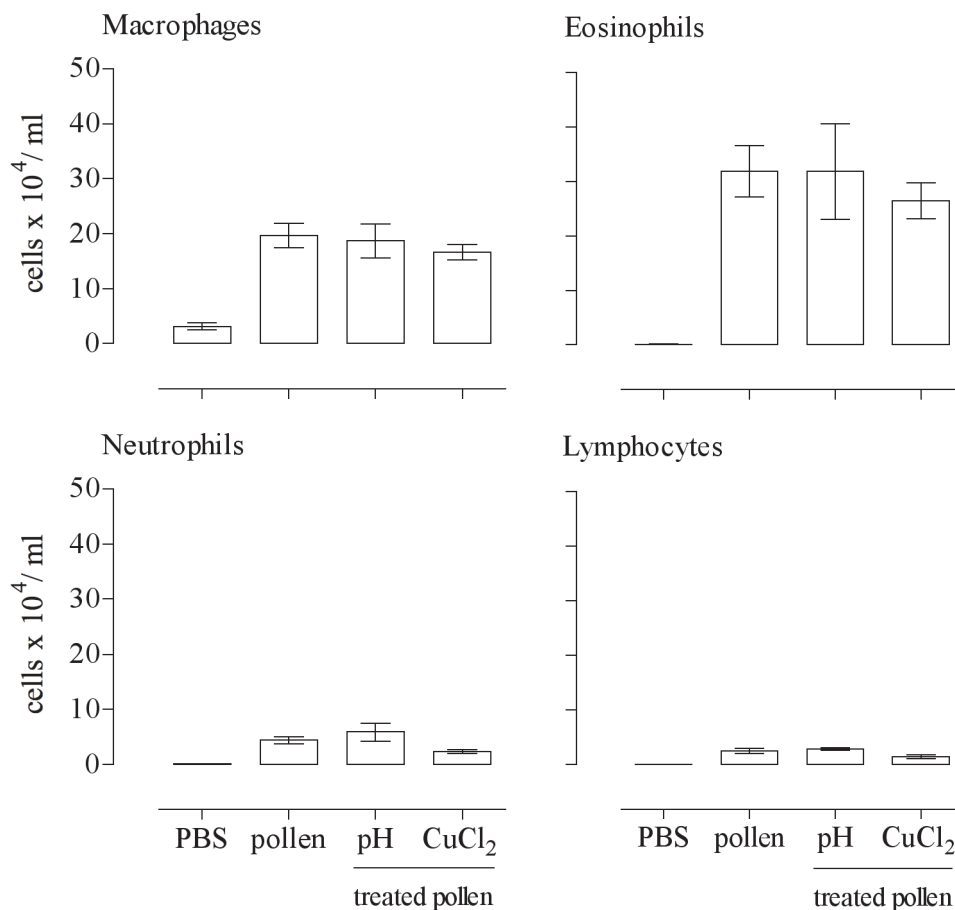


Figure 14: Cell count in BAL. Assessing total numbers for different inflammatory cell types in BAL from pollen-treated animals. Eosinophils are the most abundant cell type in BAL fluid of pollen-treated mice, followed by macrophages, neutrophils and lymphocytes. BAL fluid of PBS-treated mice consisted mainly of macrophages. No significant differences between the three groups treated with pollen. Data are expressed as means $\pm$ SEM; n=5.

Titers of Ambrosia-specific IgG1 and IgG2a showed similar results as in the previous experiment. While neither Ambrosia-specific IgG1 nor IgG2a could be detected in the sera of PBS-treated mice, all pollen-treated animals showed a definite increase in Ambrosia specific IgG1 levels (Figure 15). All pollen-treated groups showed similar high titers, with the CuCl₂-pollen group having a slightly lower and the pH-pollen group having a slightly higher titer count than the untreated pollen group. IgG2a was not detectable in pollen-treated mice (Figure 15).

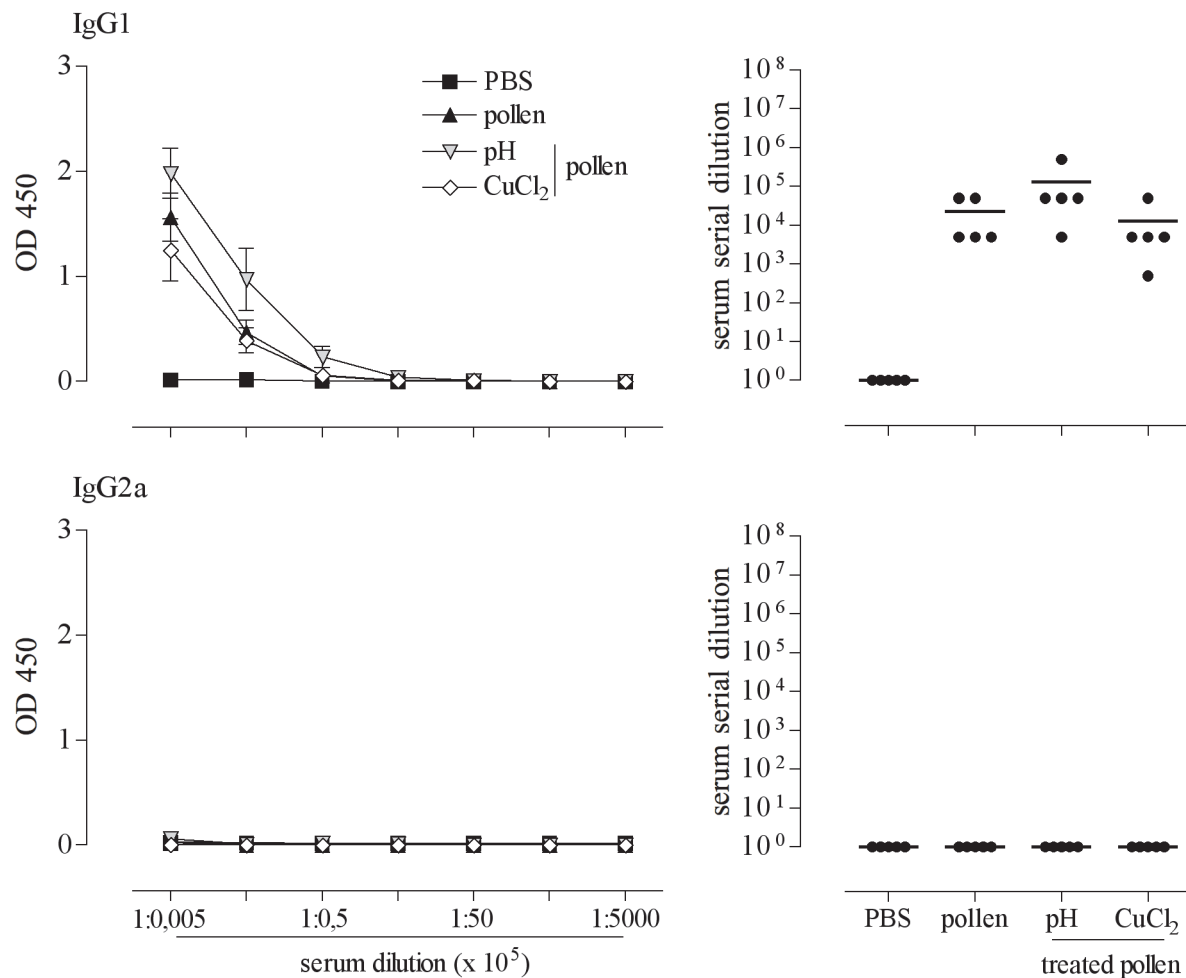
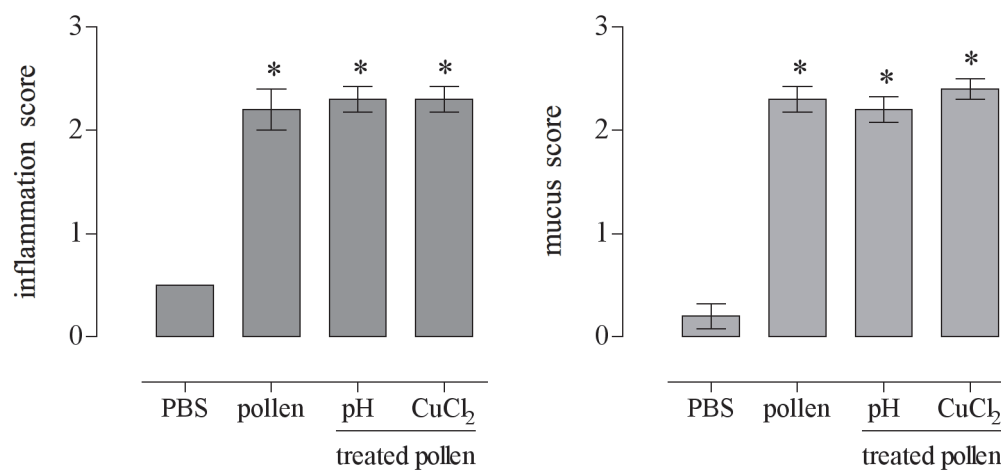


Figure 15: Serum antibody titers for Ambrosia-specific IgG1 and IgG2a comparing the possible impact of low pH and CuCl₂ on pollen sensitization. Similar IgG1 titers were detected in all pollen-treated groups with no IgG1 in PBS-treated mice. IgG2a was detectable neither in pollen- nor in PBS-treated mice. Ig-curves are shown on the left side and reciprocal titer levels on the right side. Data are expressed as mean OD₄₅₀ nm ± SEM; n = 5.

H&E stained lung sections from PBS mice showed no signs of inflammation, while lungs from pollen-treated mice had severe manifestations of inflammation with eosinophilic infiltration (Figure 16). All three groups treated with pollen showed lung inflammation with inflammatory infiltrates extending into the peripheral airways, but no difference could be detected between the groups.

Mucus production was absent in PBS-treated mice, whereas pollen-treated mice displayed abundant mucus production and a wide distribution of mucus-producing epithelial lung cells across the lungs (Figure 16). All three pollen-treated groups showed similar abundance and distribution of goblet cells reaching the peripheral airways.



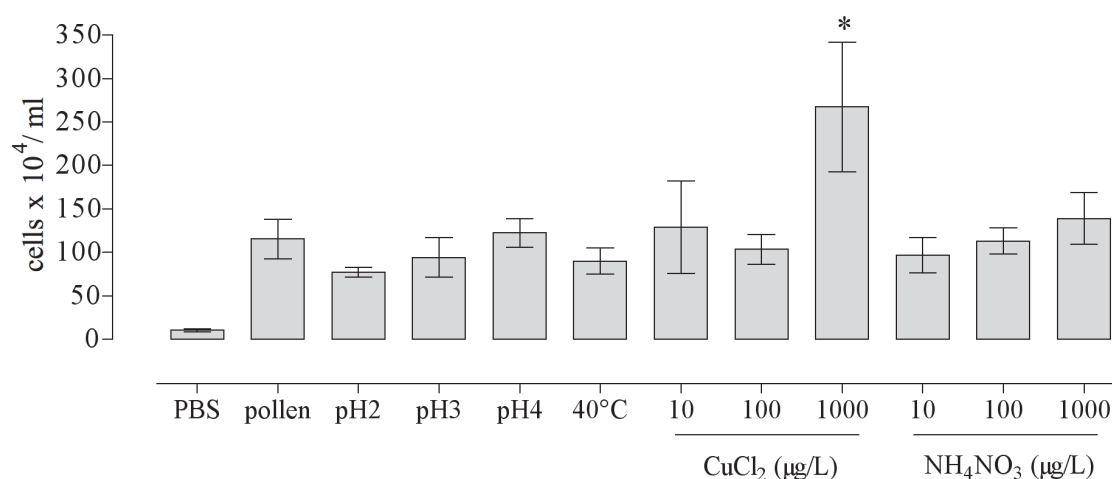
*Figure 16: Inflammation and Mucus scoring. Evaluation of inflammation and mucus production in H&E- and PAS-stained lung sections comparing the possible impact of low pH and CuCl₂ on pollen allergenicity. Significant differences comparing mice treated with PBS versus pollen-treated mice. No significant differences were detected between different pollen treatment groups. Grade 0 represents no inflammation or mucus production, while an increased grade is associated with an increase in abundance and distribution of inflammation or mucus production. Data are expressed as means ± SEM; n=5 *Significant to PBS group, Data were compared with one-way ANOVA and Bonferroni post-test, $p < 0,001$*

In conclusion, our first experimental setup to assess any possible impact of environmental factors on Ambrosia pollen allergenicity revealed no significant changes in the allergenicity between untreated pollen and pH- and CuCl₂-treated pollen.

3.2.2 Second repeat to assess possible changes of pollen allergenicity following pollen treatment with different environmental factors

We extended our studies to include a wider range of the previously tested stress conditions, and included two extra scenarios: Heat and another potential pollutant, NH_4NO_3 . Overall, in our second approach we extended the simulated environmental scenarios, and exposed pollen to heat (40°C), several low pH solutions (pH 2, 3 and 4), CuCl_2 solutions (10, 100 and $1000\mu\text{g/L}$), and NH_4NO_3 solutions (10, 100 and $1000\mu\text{g/L}$). Pollen from Allergon was incubated as previously mentioned under these new conditions and subsequently used to induce disease following our established protocol. Three weeks later animals were sacrificed and diseases evaluated. A total of $40\mu\text{g}$ of pollen per challenge was used.

All the animals treated with pollen had increased total cell count numbers in BAL, compared to mice treated with PBS (Figure 17). PBS mice had cell count numbers around 11000/ml, while cell count numbers in pollen-treated mice were highly elevated. Mice treated with pollen that was previously incubated with a solution of $1000\mu\text{g/L}$ CuCl_2 displayed the highest total cell count number in BAL (ca. 2670000 cells/ml), and showed significant differences compared to mice exposed to PBS only and to pollen treated with pH2, pH3, 40°C , $100\mu\text{g/L}$ CuCl_2 and $10\mu\text{g/L}$ NH_4NO_3 . Total BAL cell count numbers of mice treated with untreated pollen were around 1160000 cells/ml and slightly higher than of mice exposed to pH2 (770000 cells/ml), pH3 (940000 cells/ml), 40°C (900000 cells/ml), and NH_4NO_3 $10\mu\text{g/L}$ (970000 cells/ml). In the untreated pollen group, BAL cell counts were in the same range as BAL cell counts of pH4 (1220000 cells/ml), CuCl_2 $10\mu\text{g/L}$ (1290000 cells/ml), CuCl_2 $100\mu\text{g/L}$ (1040000 cells/ml) and NH_4NO_3 $100\mu\text{g/L}$ (1130000 cells/ml) treated pollen. BAL counts of mice treated with pollen exposed to NH_4NO_3 $1000\mu\text{g/L}$ (1390000 cells/ml) were slightly higher than in mice treated with normal pollen.



*Figure 17: Airway inflammation. Total cell count in BAL from mice challenged with pollen previously subjected to different environmental stress scenarios: Airway inflammation in mice challenged with pollen incubated in a CuCl₂ (1000µg/L) solution was significant higher than in animals treated with pollen exposed to pH2 and pH3, 40°C, 100µg/L CuCl₂ and 10µg /L NH₄NO₃. All pollen groups had higher significantly higher BAL cell number versus mice treated with PBS. Data are expressed as means ± SEM; n=5. *Significant to pH2 and pH3, 40°C, 100µg/L CuCl₂ and 10µg /L NH₄NO₃ groups. Data were compared with one-way ANOVA and Bonferroni post-test, $p < 0.01$.*

BAL fluid of PBS-treated mice was characterized by macrophages being the most abundant cell type (ca. 97%) with only few eosinophils (2%), neutrophils (1%) and almost no lymphocytes (Figure 18). In the pollen-treated animals, apart from 10µg/L NH₄NO₃-treated group, eosinophils represented the most abundant cell type with percentages ranging from 42% to 59%. No significant differences could be found in the distribution of eosinophils in BAL comparing all groups of mice that were treated with pollen. BAL from mice treated with native pollen consisted of 56% eosinophils and was slightly higher than in mice exposed to pollen treated with pH2 (45%), 40°C (47%) and 10µg/L NH₄NO₃ (42%) and in the same range as pH3 (57%), pH4 (59%), CuCl₂ 10µg/L (56%), CuCl₂ 100µg/L (55%), CuCl₂ 1000µg/L (55%), NH₄NO₃ 100µg/L (52%) and NH₄NO₃ 1000µg/L (54%). Macrophages were in the range of 27% to 49%, neutrophils were between 4% and 14% and lymphocytes had a low percentage between 2% and 7%.

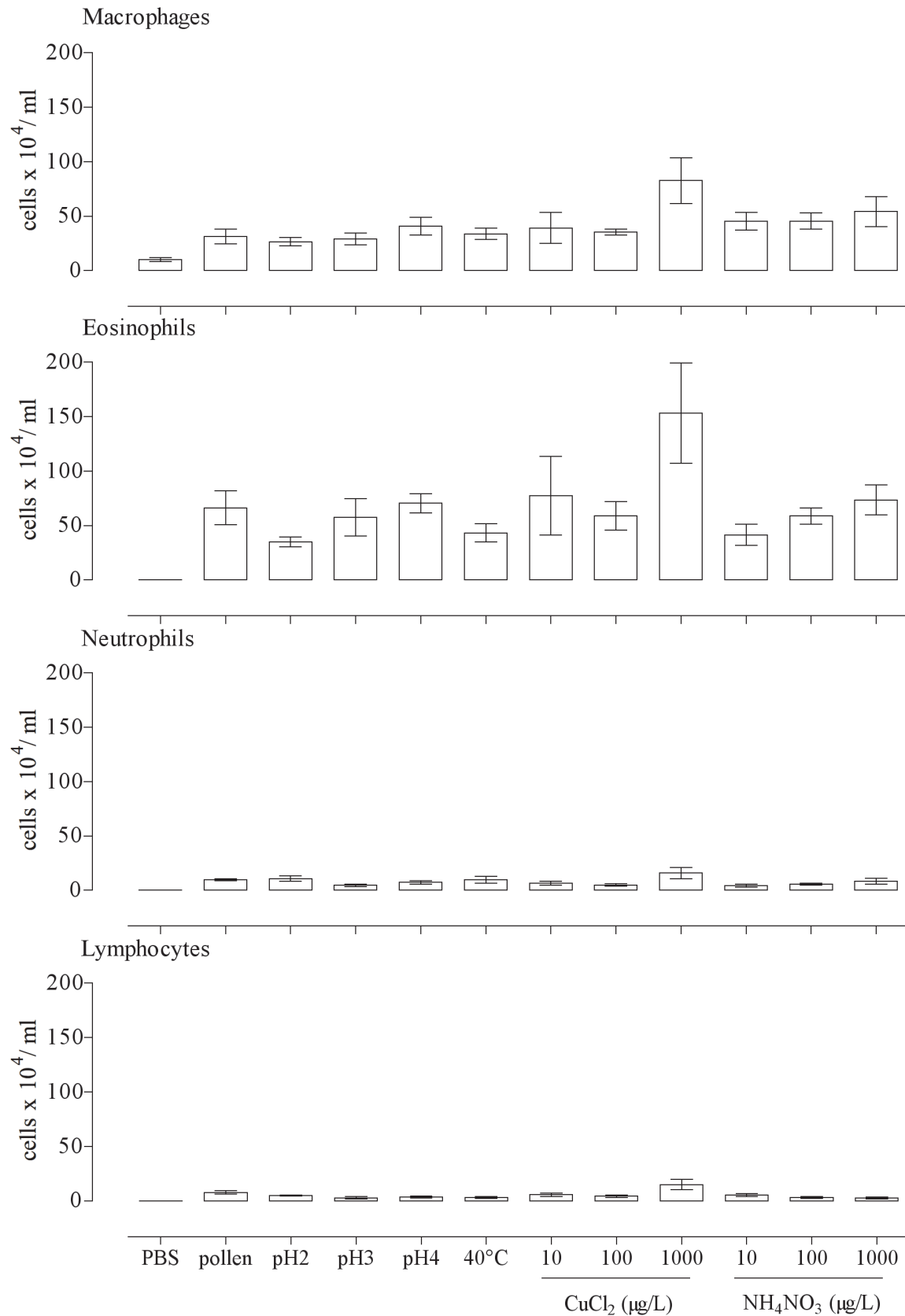


Figure 18: BAL differential cell count in mice challenged with pollen. Eosinophils are the most abundant cell type in BAL fluid in all pollen-treated mice, followed by macrophages, neutrophils and lymphocytes. BAL fluid of PBS-treated mice consisted mainly of macrophages. No significant differences could be detected in eosinophil distribution between the groups treated with pollen. Data are expressed as means $\pm$ SEM; $n=5$.

Ambrosia-specific IgG1, IgG2a and IgE were not detected in PBS-treated mice, while all pollen-treated mice had elevated IgG1 titers in their sera (Figures 19-21). IgG1 titers were similar for all pollen-treated mice; only pH2-exposed pollen had a slightly lower IgG1 titer. Ambrosia-specific IgG2a and IgE were not detectable or had very low signal. No significant differences in Ig titer levels were found between the different pollen-treated groups.

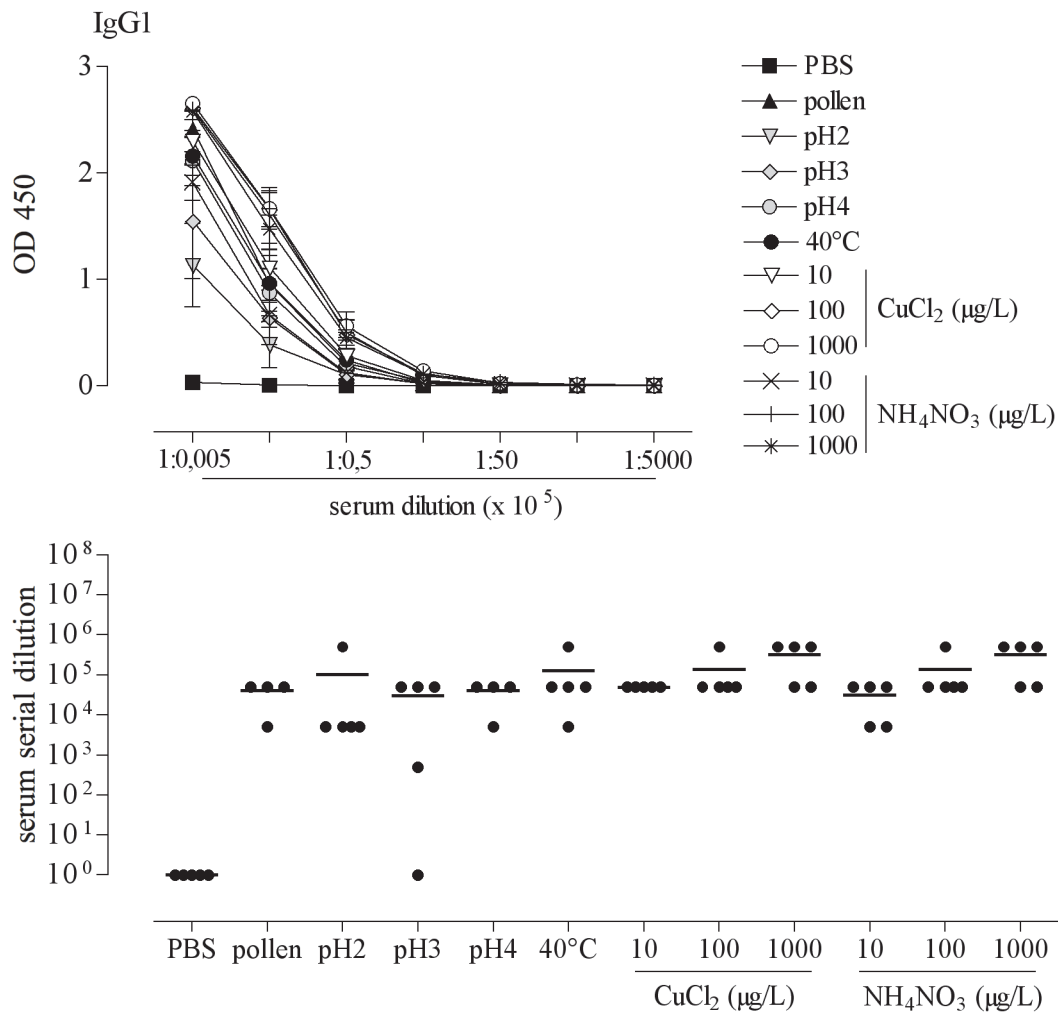


Figure 19: Serum antibody titers for Ambrosia-specific IgG1 in mice challenged with pollen subjected to different environmental scenarios. Similar IgG1 titers in all pollen-treated groups and no IgG1 in PBS-treated mice were observed. Ig-curves are shown on top and reciprocal titer levels in the lower graph. Data are expressed as mean OD_{450 nm} ± SEM; n = 5.

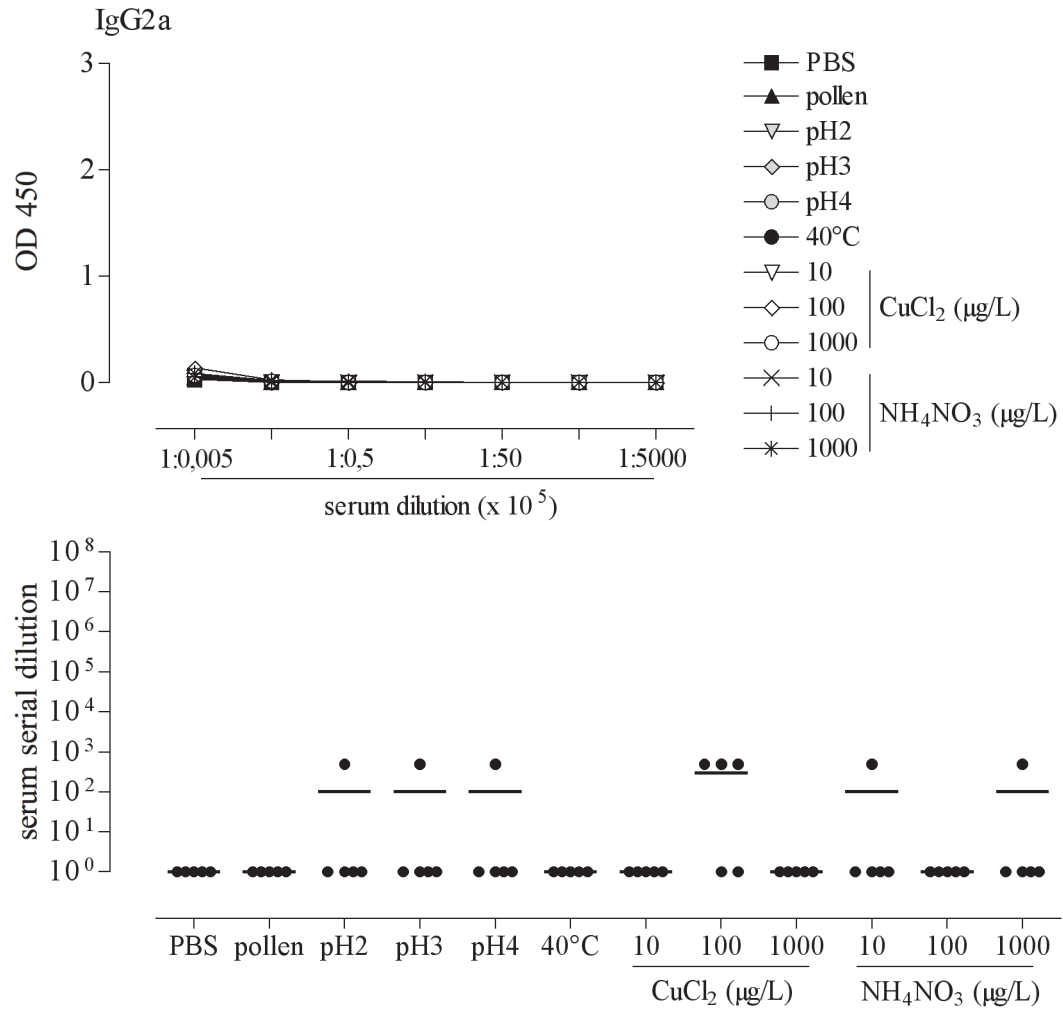


Figure 20: Serum antibody titers for *Ambrosia*-specific IgG2a in mice challenged with pollen subjected to different environmental scenarios. Low levels of IgG2a were detected in all groups. Ig-curves are shown on top and reciprocal titer levels in the lower graph. Data are expressed as mean OD_{450 nm} ± SEM; n = 5.

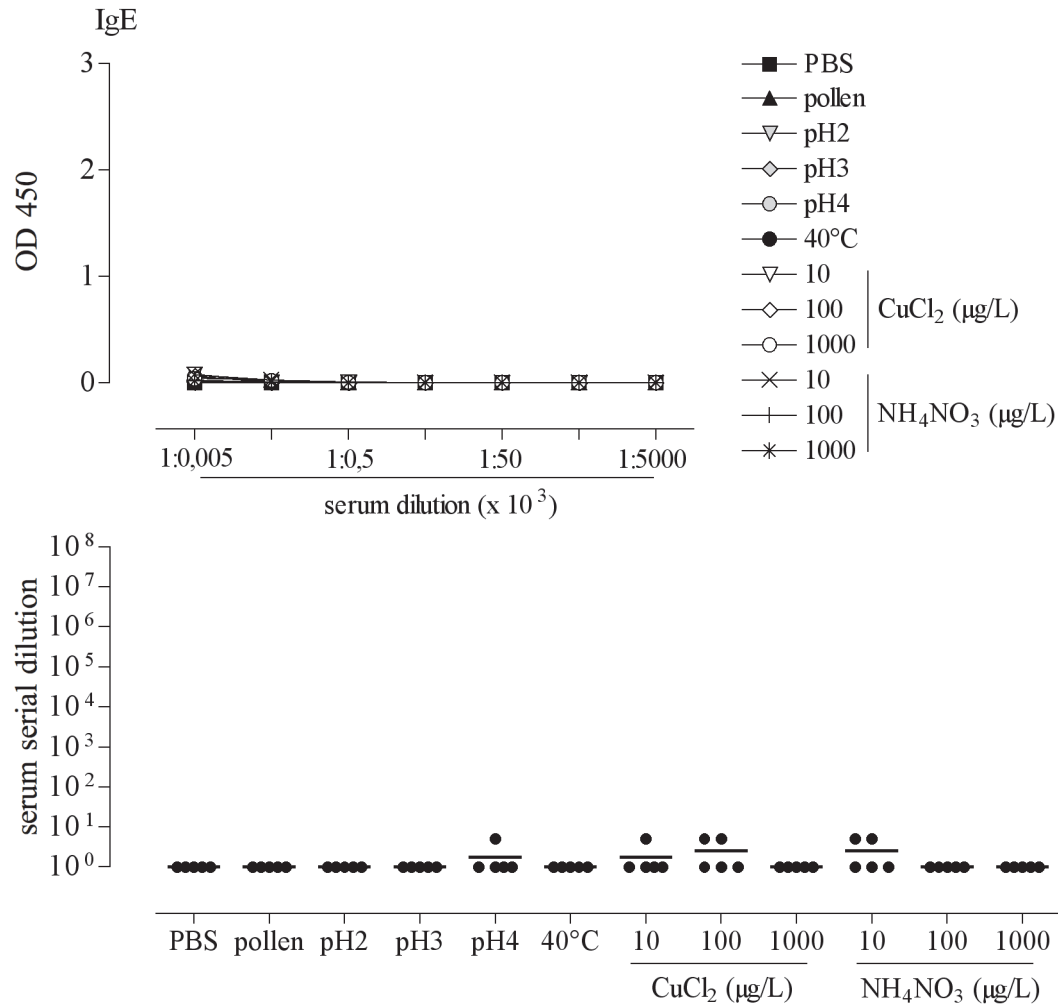
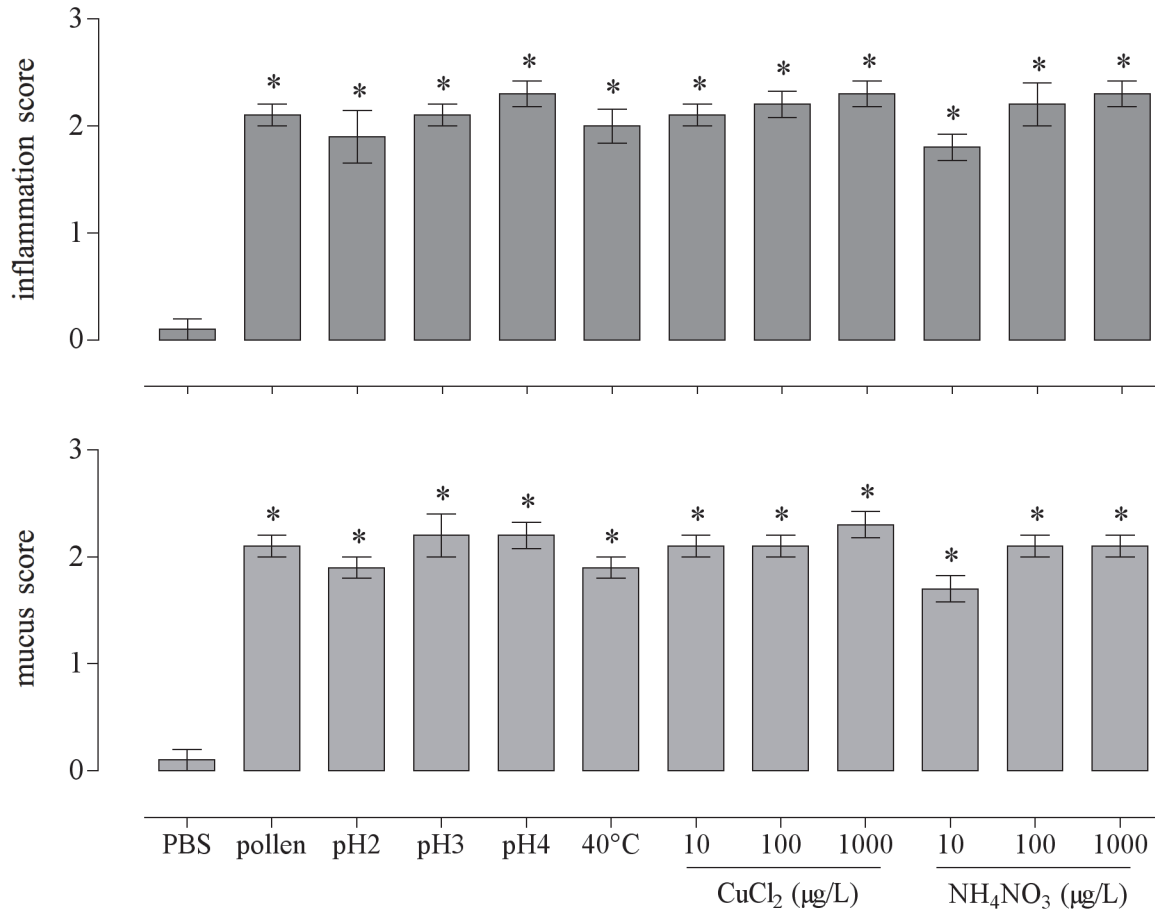


Figure 21: Serum antibody titers for Ambrosia-specific IgE in mice challenged with pollen subjected to different environmental scenarios. IgE was not detectable in pollen or in PBS-treated mice. Ig-curves are shown on top and reciprocal titer levels on the bottom graph. Data are expressed as mean OD_{450 nm} ± SEM; n = 5.

H&E stained lung sections from PBS mice showed no signs of inflammation, whereas lungs from pollen-treated mice displayed severe manifestations of inflammation with eosinophilic infiltration (Figure 22). All groups treated with pollen showed lung inflammation with inflammatory infiltrates extending to the peripheral airways, but no significant difference could be detected between the pollen groups.

Mucus production was absent or very little in PBS-treated mice, whereas lungs of pollen-treated mice displayed abundant mucus production and wide distribution of mucus producing epithelial lung cells across the lungs (Figure 22). All pollen-treated groups showed similar

abundance and distribution of goblet cells, which were extending up into the peripheral airways.



*Figure 22: Scoring of inflammation and mucus production in H&E- and PAS-stained lung sections in mice challenged with pollen subjected to environmental scenarios. All pollen-treated animals had a significant score for inflammation and mucus production compared to PBS pollen-treated mice. However, no significant difference between different pollen-treated groups could be detected. Grade 0 represents no inflammation or mucus production, while an increased grade is associated with an increase in abundance and distribution of inflammation or mucus production. Data are expressed as means $\pm$ SEM; $n=5$. *Significant to PBS group. Data were compared with one-way ANOVA and Bonferroni post-test, $p < 0,001$.*

In conclusion, exposure of Ambrosia pollen to our tested environmental scenarios was able to induce disease. However, no significant differences in airway inflammation, immune response, and lung inflammation were observed between treated and untreated pollen. Interestingly, only incubation of pollen with a high concentration of CuCl₂ (1000µg/L) revealed significant differences in total cell count compared to other pollen treatment groups. Moreover, as an observation of interest, total cell count was generally higher than in previous

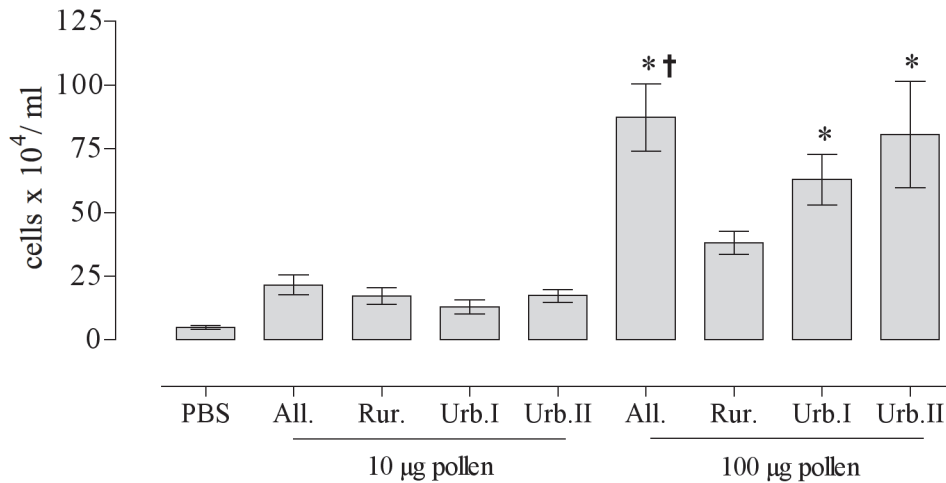
experiments. Percentages of eosinophils were similar in all pollen-treated groups with only slight differences and no definite differences found in the level of Ambrosia-specific Ig titers. Mucus production and lung inflammation were similar for all pollen-treated groups and showed no evident differences.

3.2.3 Comparison of pollen allergenicity for company-derived pollen and collected rural and urban pollen

Next, we hypothesized that pollen from Allergon was altered during purification procedures and this may interfere with the allergenicity properties of the pollen. Therefore we investigated the allergenicity of company-purchased pollen (Allergon) and pollen from locally harvested rural and urban Ambrosia populations. Pollen from Ambrosia plants was collected. First population plants were located in a rural area, while two other populations from which pollen was harvested were growing in an urban environment. Pollen was carefully collected and used subsequently to induce disease in our mouse asthma model.

In our first comparison experiment we used company pollen (Allergon), pollen from rural area (rural pollen) and urban pollen samples from two different urban areas (urban pollen I and urban pollen II). A total of 10 μ g and 100 μ g doses of pollen per challenge were used.

Administration of low doses of pollen (10 μ g) induced a mild increase in total BAL cell counts, compared to PBS (Figure 23). BAL total cell count in PBS-treated mice was around 50000/ml, whereas BAL cell counts displayed a similar value after treatment with 10 μ g Allergon pollen (220000 cells/ml), rural pollen (170000 cells/ml) and urban pollen II (170000 cells/ml), with a slightly smaller increase following treatment with 10 μ g urban I pollen (120000 cells/ml). High dosage of pollen (100 μ g) induced a marked increase in BAL total cell counts versus PBS group (Figure 23). Allergon and both urban pollen administration led to significant increase of cell count numbers compared to PBS-treated mice, whereas treatment with rural pollen resulted in visible but not significant increase of BAL cell count numbers. BAL cell count numbers of mice treated with Allergon pollen were around 870000 cells/ml, similar to urban II pollen (810000 cells/ml), slightly higher than urban pollen I (630000 cells/ml), and significantly higher than rural pollen (380000 cells/ml).



*Figure 23: Airway inflammation. Total cell count of BAL revealing airway inflammation in animals challenged with Allergon versus rural versus urban pollen. Treatment with high doses of company-derived pollen (Allergon 100µg) and both urban pollen (urban pollen I 100µg and urban pollen II 100µg) induced a significantly increase in total cell counts in BAL compared to PBS-treated mice (*significant to PBS group; $p < 0,01$). Mice treated with 100µg of Allergon pollen, compared to mice treated with rural pollen had significantly increased BAL cell numbers (†significant to rural pollen group; $p < 0,05$). All.: Allergon; Rur.: rural; Urb.I: urban I; Urb.II: urban II pollen. Data are expressed as means $\pm$ SEM; $n=8$, (one-way ANOVA and Bonferroni post-test).*

BAL of PBS-treated mice was characterized by macrophages being the most abundant cell type (ca. 97%) with only few eosinophils (2%) and almost no neutrophils and lymphocytes (Figure 24). Low doses of pollen (10µg) resulted in an increased number of eosinophils, but with macrophages remaining the most abundant cell type at around 58% to 65% of the BAL cell composition. Eosinophil percentages in BAL ranged from 28% to 39%, with rural pollen showing the lowest (28%) and urban II pollen showing the highest eosinophil (39%) percentage. By comparison BAL of Allergon and urban I pollen-treated mice showed a percentage of eosinophils of 31% and 32% respectively. Neutrophil percentages were low at around 2% and lymphocytes had a similar distribution between 1% and 5% in all groups treated with 10µg pollen.

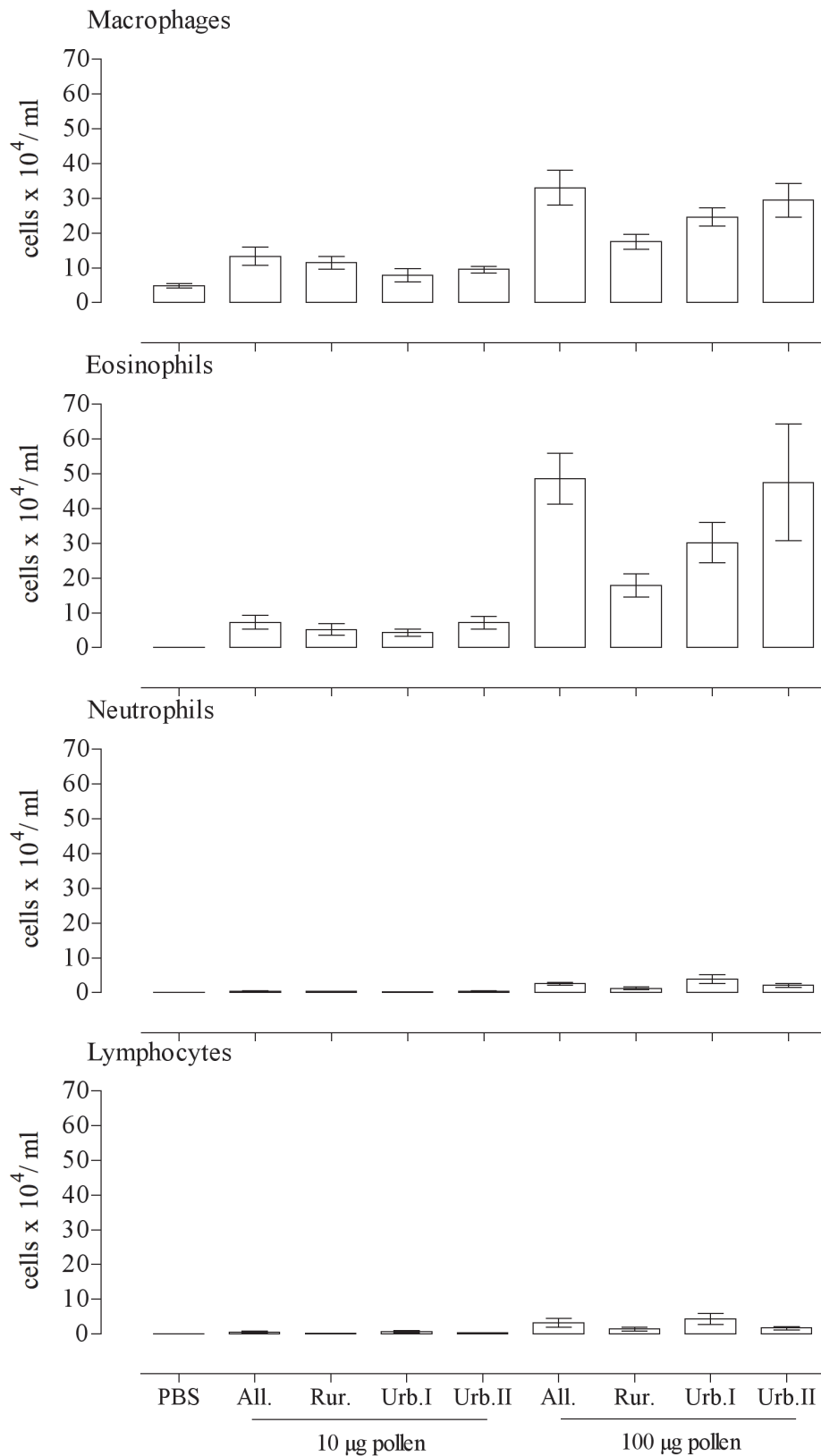


Figure 24: BAL differential cell count in mice challenged with different *Ambrosia* pollen. Macrophages were the most abundant cell type in BAL fluid in all mice treated with low pollen doses (10µg), followed by eosinophils. In mice treated with high pollen doses (100µg) eosinophils was the most abundant cell type in BAL fluid in all mice, except for mice treated with rural pollen, in which macrophages were more abundant than eosinophils. BAL fluid of PBS-treated mice consisted mainly of macrophages. All.: Allergon; Rur.: rural; Urb.I: urban I; Urb.II: urban II pollen. Data are expressed as means $\pm$ SEM; $n = 8$.

As for the mice that received 100µg of pollen, in Allergon and both urban pollen groups, eosinophils represented the most abundant cell type (Figure 24). Eosinophil percentages ranged from 46% to 56% and macrophages between 38% and 42%. In contrast, mice treated with rural pollen displayed more macrophages than eosinophils in their BAL fluid (48%, compared to 45% respectively).

Mice treated with 100µg Allergon pollen displayed the highest percentage of eosinophils (56%), followed by urban II pollen (53%), urban I pollen (46%) and rural pollen (45%). In all groups treated with 100µg pollen, neutrophils rates were between 3% to 5% and lymphocytes ranged from 3% to 7%.

As expected, mice treated with PBS had no detectable Ambrosia-specific IgG1, IgG2a or IgE (Figures 25-27). Groups treated with 10µg of pollen had a minor increase in Ambrosia-specific IgG1 titer in their sera, whereby treatment of 10µg of urban II pollen led to a slightly higher IgG1 titer than all the other 10µg pollen treatment. No IgG2a and IgE were detectable in all groups treated with 10µg pollen. Treatment with 100µg pollen resulted in a higher increase of Ambrosia-specific IgG1 titer, which was similar in all groups. While groups treated with 100µg of Allergon pollen did not show Ambrosia-specific IgE, mice treated with 100µg of rural and urban pollen had a minor titer of Ambrosia-specific IgE. No IgG2a was detected in any groups treated with 100µg pollen.

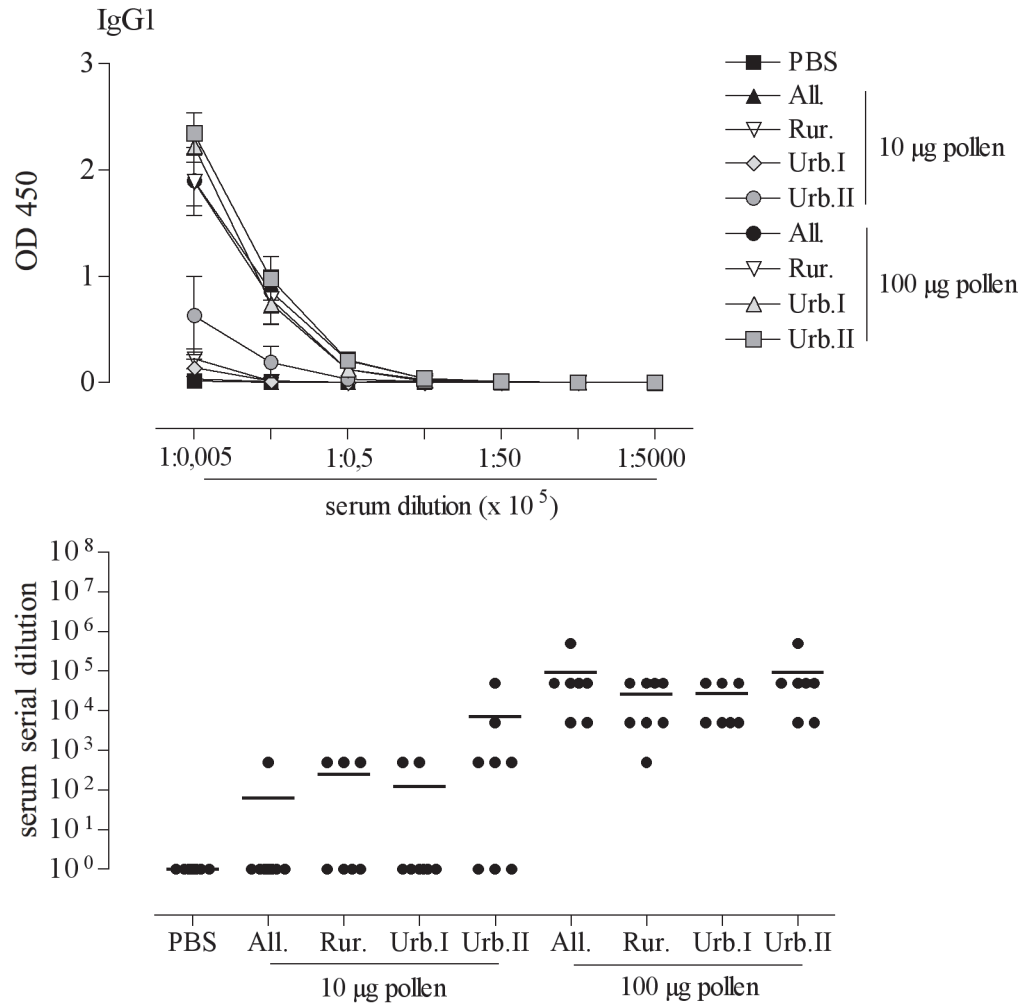


Figure 25: Serum antibody titers for Ambrosia-specific IgG1 in mice immunized to Allergon and local collected pollen. Groups, treated with 10µg of pollen had minimal increase of Ambrosia-specific IgG1, with a slight increase for urban II group when compared to other pollen groups. Treatment with 100µg pollen resulted in a higher Ig titer when compared to 10µg groups with no significant difference between 100µg groups. Ig-curves are shown on top and reciprocal titer levels in the bottom graph. All.: Allergon; Rur.: rural; Urb.I: urban I; Urb.II: urban II pollen. Data are expressed as mean OD_{450 nm} ± SEM; n = 8.

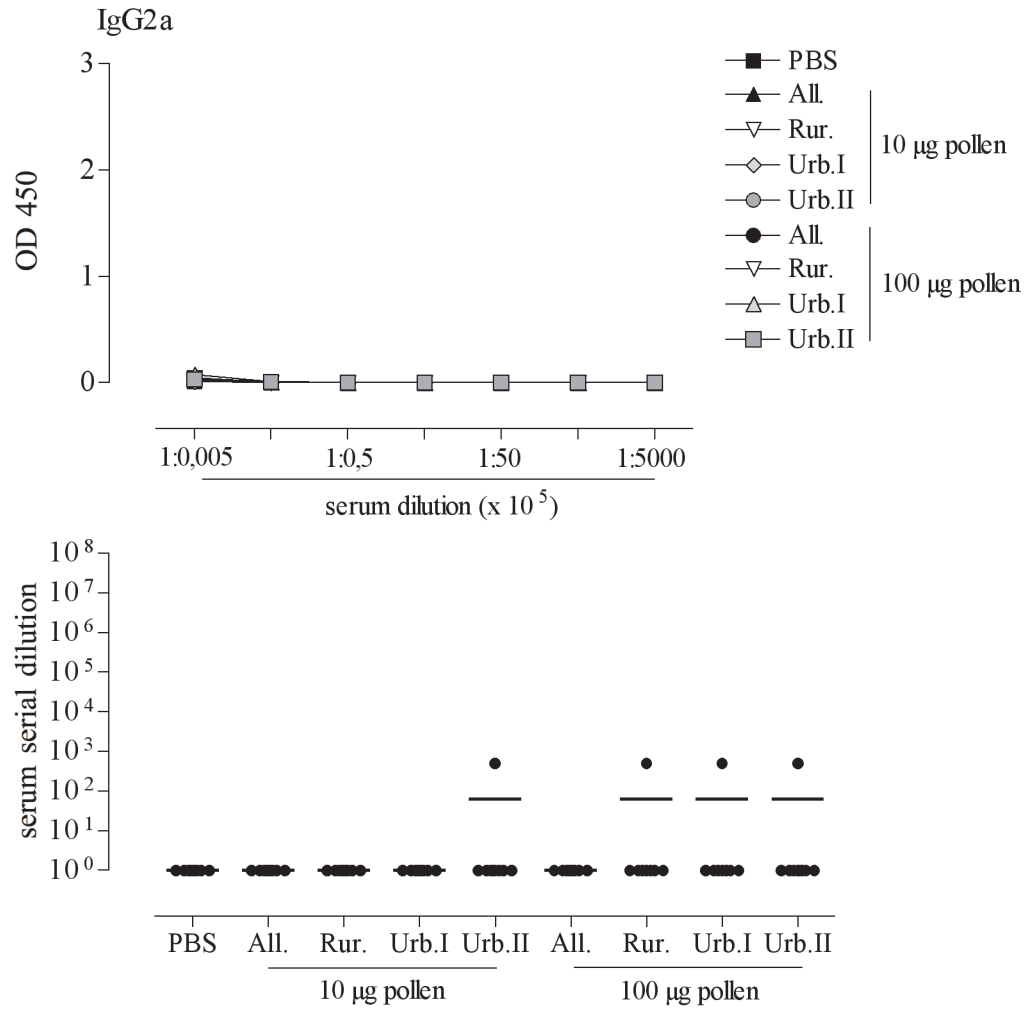


Figure 26: Serum antibody titers for *Ambrosia*-specific IgG2a in mice immunized to Allergon and local collected pollen. IgG2a was not detectable in pollen or in PBS-treated mice. Ig-curves are shown on top with the reciprocal titer levels in the bottom graph. All.: Allergon; Rur.: rural; Urb.I: urban I; Urb.II: urban II pollen. Data are expressed as mean OD_{450 nm} ± SEM; n = 8.

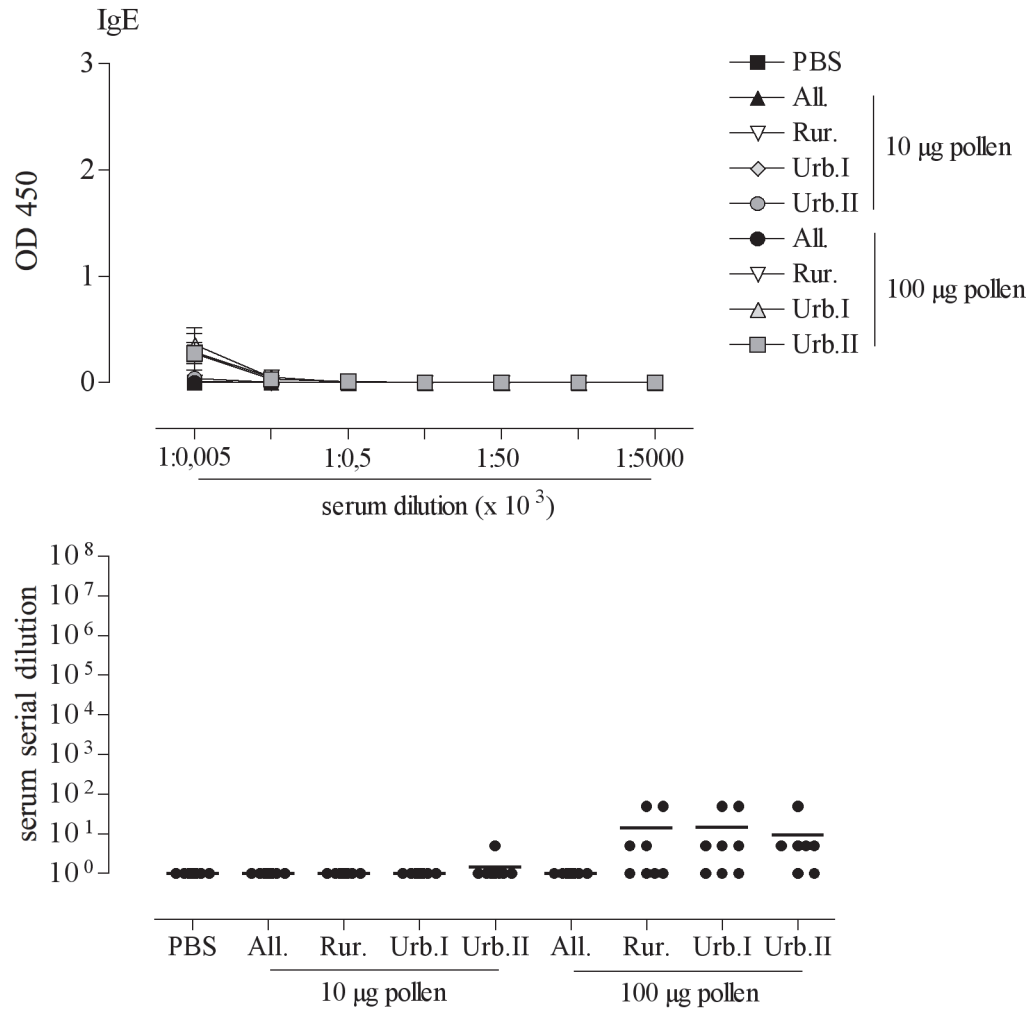


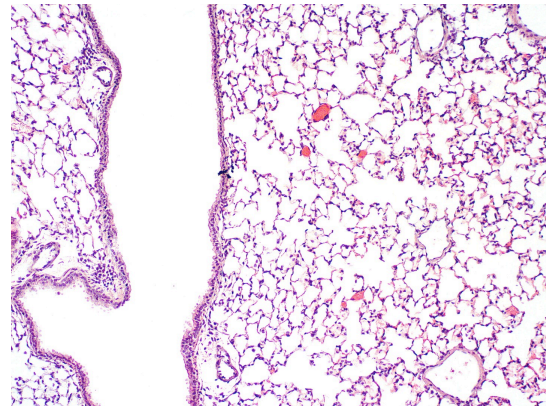
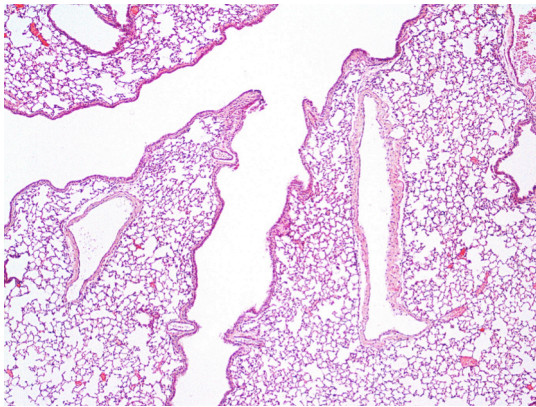
Figure 27: Serum antibody titers for Ambrosia-specific IgE in mice immunized to Allergon and local collected pollen. No IgE was detectable in PBS-, and 10µg pollen-treated groups. Interestingly, treatment with 100µg pollen induced a slight increase of Ambrosia-specific IgE, with the exception for Allergon pollen. Ig-curves are shown on top and reciprocal titer levels in the bottom graph. All.: Allergon; Rur.: rural; Urb.I: urban I; Urb.II: urban II pollen. Data are expressed as mean OD₄₅₀ nm ± SEM; n = 8.

Evaluation of H&E-stained lung sections showed no inflammatory infiltrates in lungs of PBS-treated mice (Figures 28 & 29). Lungs of mice that had received 10µg pollen per challenge, showed inflammatory infiltrates mainly in and around central airways with minor inflammation in the middle airways. Administration of 100µg pollen induced a more severe inflammation, involving more inflammatory infiltrates that were also present in and around the peripheral airways.

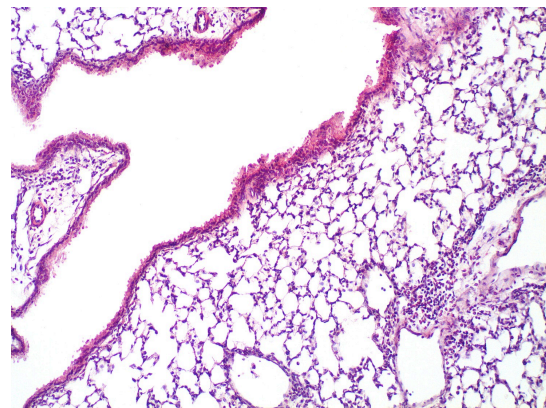
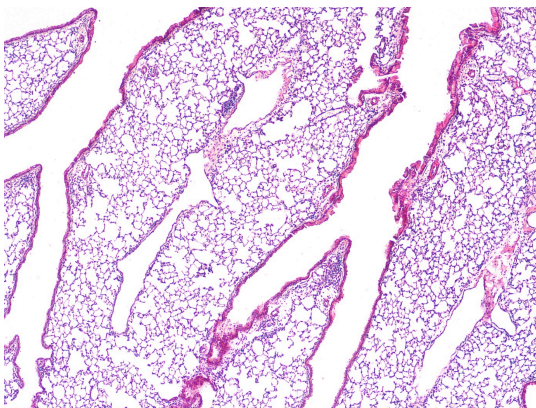
PBS

4X

10X



10 μ g pollen



100 μ g pollen

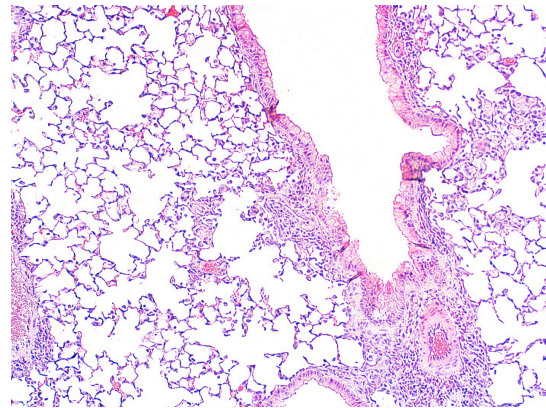
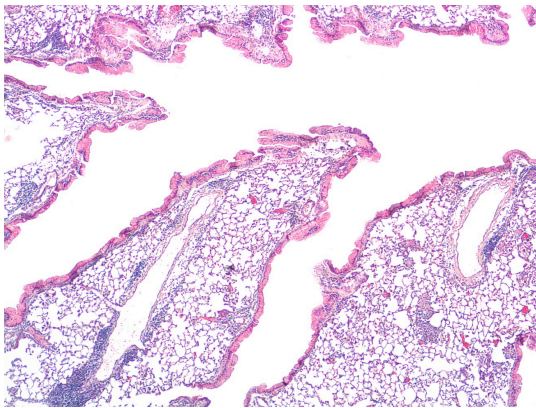
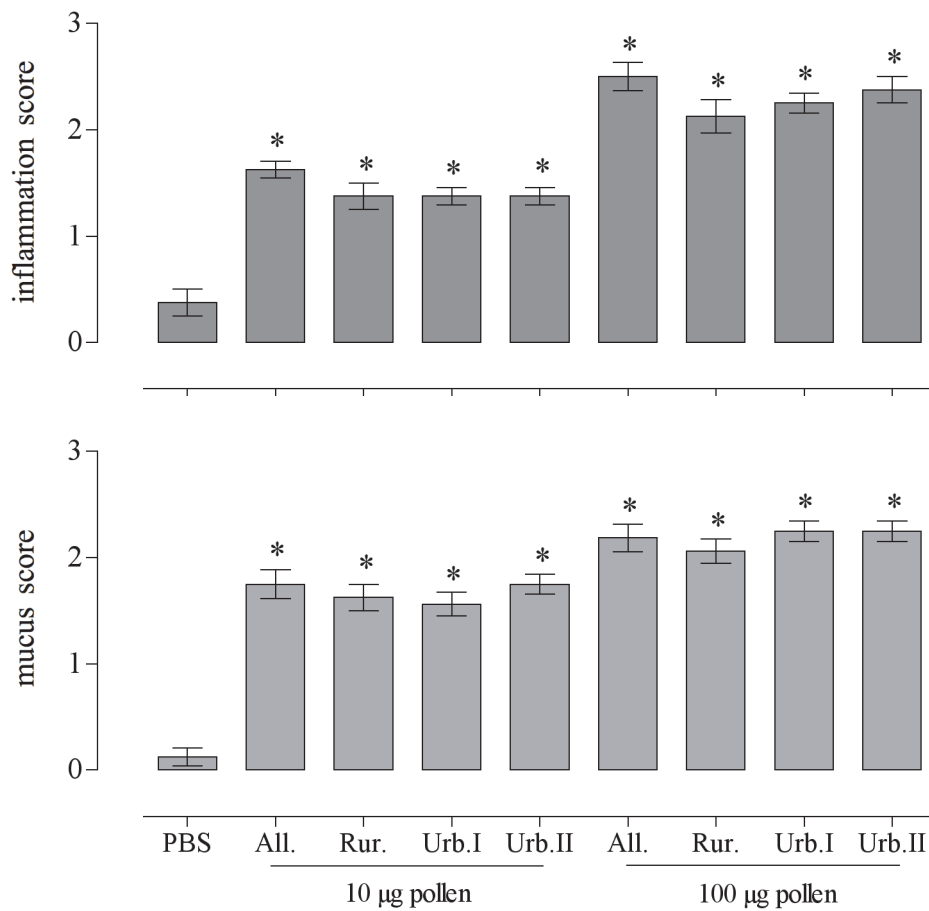


Figure 28: H&E staining of lung sections in mice immunized to Allergon and local collected pollen. Representative photomicrographs (4X and 10X) of H&E-stained lung sections showing lung inflammation in PBS- and pollen- (10 μ g and 100 μ g) challenged mice. No inflammatory infiltrates could be observed in lungs of PBS-treated mice. Lungs of mice treated with a dose of 10 μ g pollen showed inflammatory infiltrates mainly in and around central airways and weak inflammation at middle airways level. Administration of 100 μ g pollen induced a more severe inflammation, represented by inflammatory infiltrates that extend up to the periphery.

PAS-stained lung sections from mice treated with PBS displayed none or very few mucus-producing cells (Figures 29 & 30). Administration of pollen (10 μ g and 100 μ g) induced mucus hypersecretion. Lungs from mice that received 10 μ g pollen were characterized by mucus secretion in central and middle airways, while treatment with 100 μ g pollen resulted in heavy mucus hypersecretion and presence of mucus producing goblet cells in the periphery.

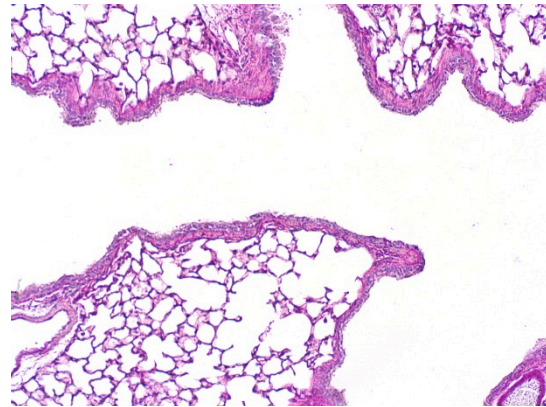
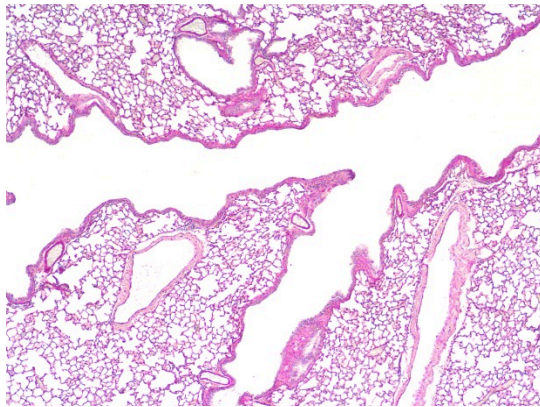


*Figure 29: Scoring of inflammation and mucus production in H&E- and PAS-stained lung sections in mice challenged with Allergon and local collected pollen (10 and 100 μ g). All pollen-treated animals had a significant score for inflammation and mucus production compared to PBS-treated mice. Mice treated with 100 μ g pollen showed more severe lung inflammation and mucus hypersecretion compared to mice treated with 10 μ g, but no significant difference between different pollen-treated groups was detected. Grade 0 represents no inflammation or mucus production, while an increased grade is associated with an increase in abundance and distribution of inflammation or mucus production. All.: Allergon; Rur.: rural; Urb.I: urban I; Urb.II: urban II pollen. Data are expressed as means $\pm$ SEM; n = 8. *Significant to PBS group. Data were compared with one-way ANOVA and Bonferroni post-test, $p < 0,001$.*

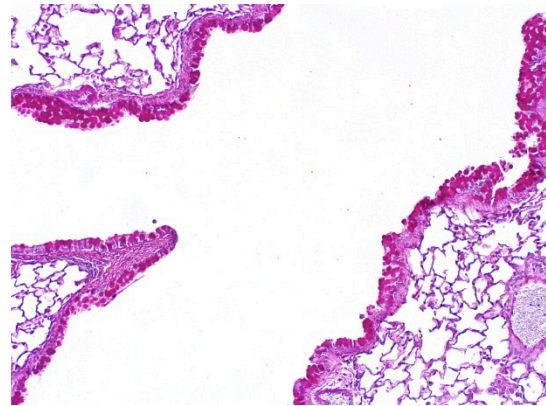
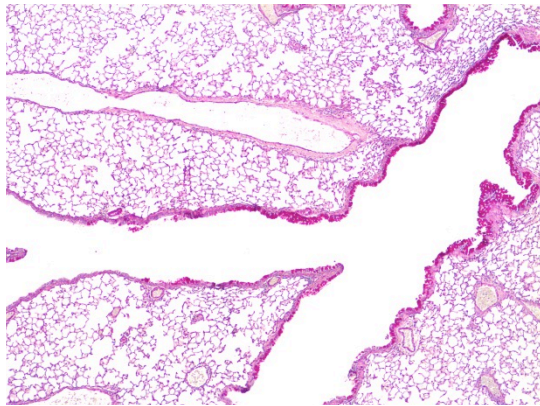
PBS

4X

10X



10 μ g pollen



100 μ g pollen

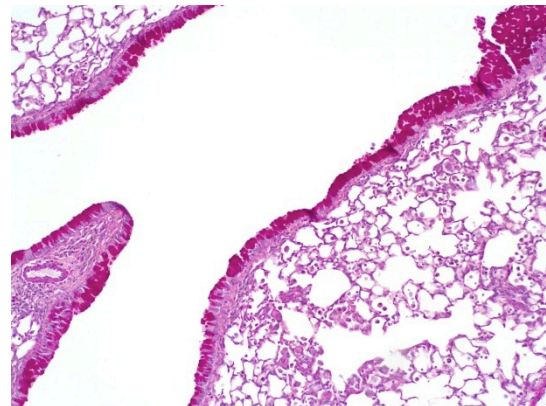
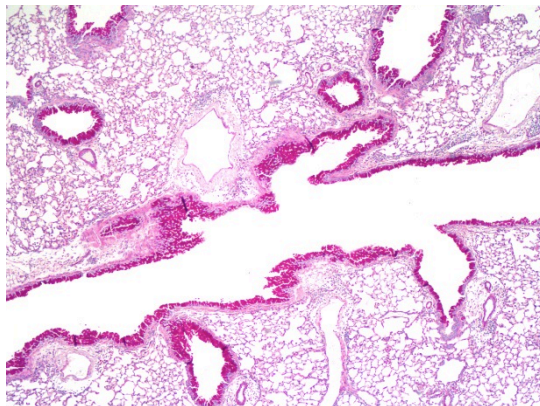


Figure 30: Mucus hypersecretion in mice immunized to Allergon and local collected pollen (10 and 100 μ g). Representative photomicrographs (4X and 10X) of PAS-stained lung sections showing mucus hypersecretion in PBS and pollen (10 μ g and 100 μ g) challenged mice. No mucus hypersecretion could be observed in lungs of PBS-treated mice. Lungs of mice treated with a dose of 10 μ g pollen showed mucus-producing goblet cells mainly in and around central airways and weak mucus secretion at middle airways level. Administration of 100 μ g pollen induced more abundant mucus secretion, represented by goblet cells that extend up to the periphery.

Comparing company-derived pollen and pollen collected from rural and urban areas, revealed significant differences in pollen allergenicity. Treatment with rural pollen in mice induced a weaker allergic response compared to the response observed with Allergon and urban pollen. Although administration of low pollen doses (10 μ g) showed no significant differences in BAL cell count numbers between all pollen-treated groups, administration of high pollen doses (100 μ g) resulted in distinguishable findings. Compared to PBS-treated mice, treatment with Allergon and both urban pollen led to significantly increased BAL cell count numbers, while BAL cell count numbers were only slightly elevated following rural pollen treatment.

In both administered pollen doses, Allergon pollen and urban II pollen treated groups displayed a higher percentage of eosinophils, compared to rural pollen. In the groups of mice treated with high dosages of Allergon and urban pollen, animals treated with 100 μ g of rural pollen had the lowest percentages of eosinophils in BAL. Moreover, mice that received rural pollen were the only pollen group where macrophages represented the most abundant cell type.

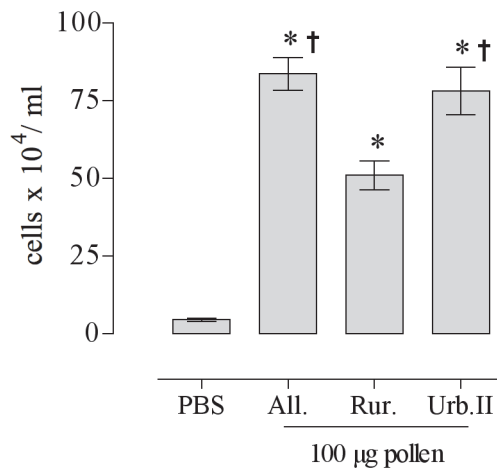
No distinct differences in Ig levels, abundance and distribution of inflammatory infiltrates and mucus hypersecretion in lungs could be detected between the different pollen treated groups. Compared to PBS treatment, IgG1 titers were clearly elevated in all mice following treatment with high pollen dose, and slightly elevated in low pollen dose groups. IgG2a was not detectable in any group and only very low IgE levels were observed following treatment with high doses of collected pollen (not Allergon). All groups treated with pollen displayed evident manifestation of lung inflammation, with inflammatory infiltrates and mucus hypersecretion. Mice treated with 100 μ g pollen displayed more severe lung inflammation, with inflammatory infiltrates, mucus hypersecretion, and mucus producing goblet cells present also in the peripheral airways.

3.2.4 Second repeat to investigate the allergenicity of company-derived pollen versus rural and urban collected pollen

To confirm possible differences in the allergenicity of company-derived or collected rural and urban pollen, we repeated the last experiment.

Because application of the high pollen dosage (100 μ g) had the most relevant results, with differences in between company-derived, rural and urban pollen allergenicity, we used exclusively 100 μ g as pollen dose for the challenge. Due to shortage of pollen quantity urban pollen I group was excluded from the experiments.

Administration of pollen induced a significant increase in total BAL cell numbers, compared to PBS-treated mice; with BAL total cell counts around 45000/ml (Figure 31). BAL cell counts following treatment with Allergon pollen (840000 cells/ml) and urban II pollen (780000 cells/ml) were significantly higher than BAL cell count numbers following treatment with rural pollen (510000 cells/ml).



*Figure 31: Airway inflammation. Total cell count of BAL revealing airway inflammation in animals challenged with Allergon versus rural versus urban pollen. Treatment with all pollen types induced a significantly increase in total cell counts in BAL compared to PBS-treated mice (*significant to PBS group; $p < 0,001$). Mice treated with Allergon or urban pollen had significantly increased BAL cell numbers compared to mice treated with rural pollen (†significant to rural pollen group; $p < 0,05$). All.: Allergon; Rur.: rural; Urb.II: urban II pollen. Data are expressed as means $\pm$ SEM; $n = 8$. Data were compared with one-way ANOVA and Bonferroni post-test.*

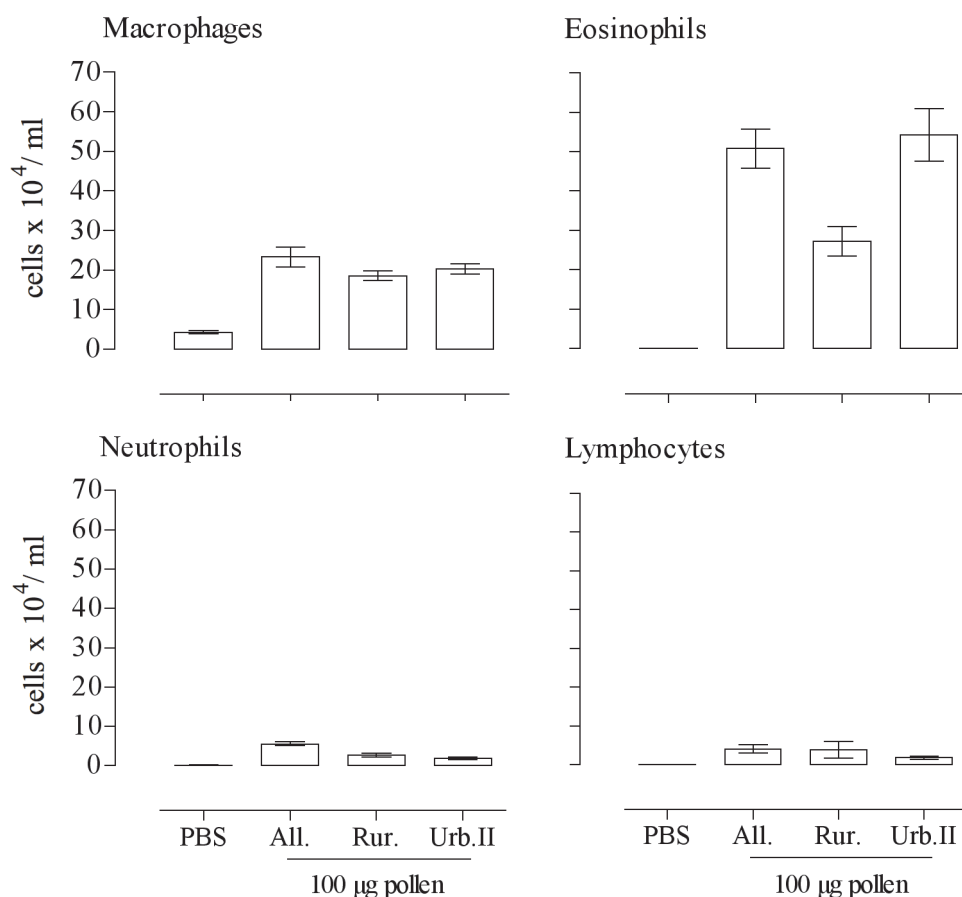


Figure 32: BAL differential cell count in mice following treatment with different pollen types. Eosinophils are the most abundant cell type in BAL fluid in all pollen-treated mice, followed by macrophages, and very low numbers of neutrophils and lymphocytes. Mice treated with urban pollen showed significantly more eosinophils in BAL, compared to mice treated with rural pollen (68% versus 51%). BAL fluid of PBS-treated mice consisted mainly of macrophages. All.: Allergon; Rur.: rural; Urb.II: urban II pollen. Data are expressed as means $\pm$ SEM; $n = 8$.

BAL of PBS-treated mice consisted mainly of macrophages (95%) and only few eosinophils, neutrophils and lymphocytes (each around 2%; Figure 32). In all pollen-treated mice, eosinophils were the most abundant cell type, representing between 51% and 68% of BAL cells. Administration of urban pollen resulted in the highest concentration of eosinophils in BAL (68%), which was significantly increased when compared to treatment with rural pollen (51%). The percentage of eosinophils in the BAL composition of mice treated with Allergon pollen was right between urban and rural pollen treatment at 60%. Macrophages represented, in all pollen-treated groups between 28% and 37% of the BAL composition, and the rest consisted of small percentages of neutrophils and lymphocytes (between 2% and 7%).

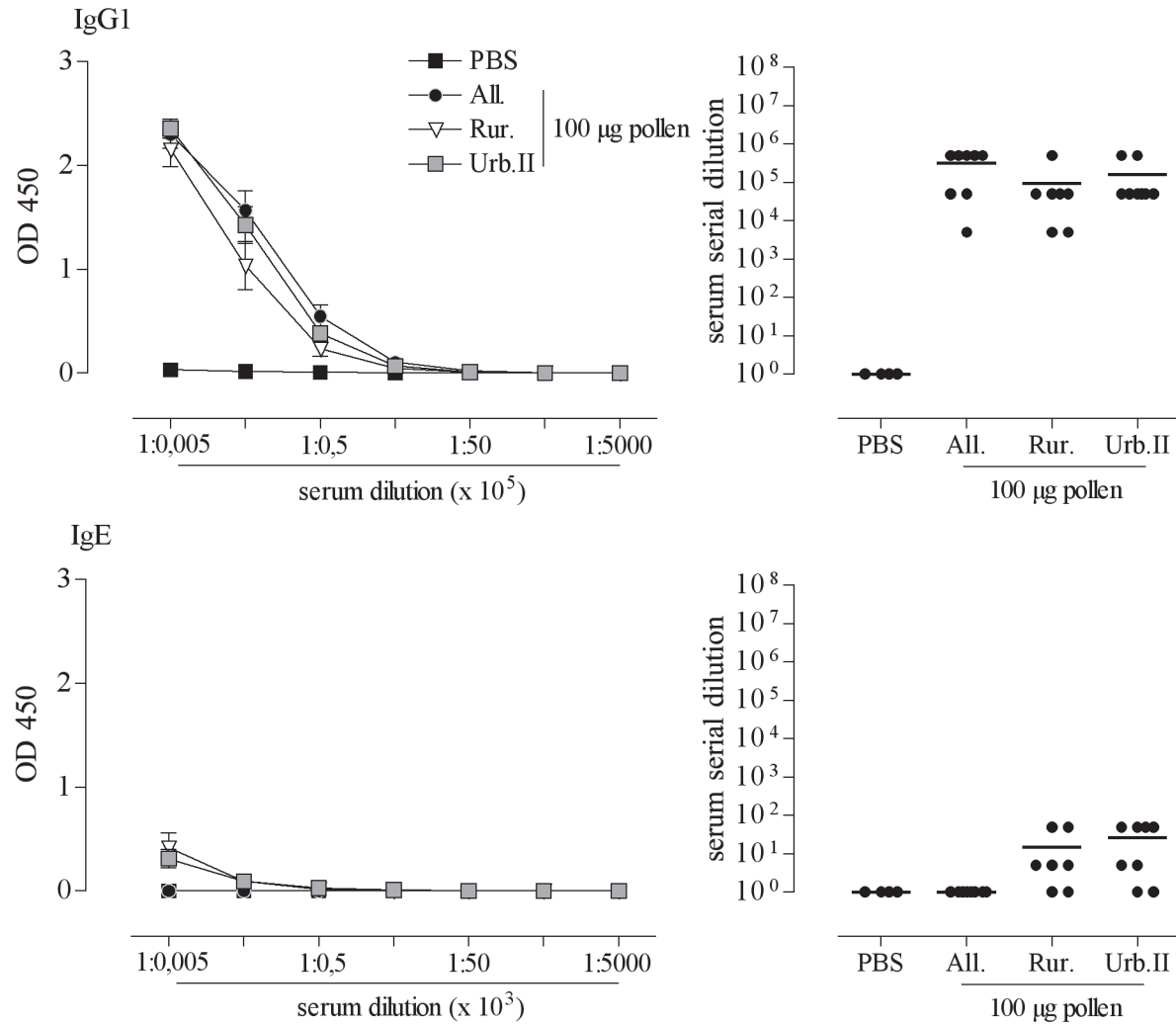


Figure 33: Serum antibody titers for Ambrosia-specific IgG1 and IgE in mice immunized to Allergon and local collected pollen. Treatment with all pollen types resulted in clearly elevated IgG1 titers, while only treatment with collected pollen types resulted in slightly increased IgE titers. No Ambrosia-specific IgG1 and IgE were detected in PBS-treated mice. Ig-curves are shown on the left and reciprocal titer levels on the right side. All.: Allergon; Rur.: rural; Urb.I: urban I; Urb.II: urban II pollen. Data are expressed as mean OD_{450 nm} $\pm$ SEM; n = 8.

Mice treated with PBS showed no detectable Ambrosia-specific IgG1 or IgE (Figure 33). High titer of Ambrosia-specific IgG1 could be detected in the sera from all the mice treated with pollen. Nevertheless titer levels were similar in all groups. Moreover, treatment with collected rural and urban pollen resulted in the detection of a low Ambrosia-specific IgE titer, while no IgE was detected in mice challenged with Allergon pollen.

We observed similar results in our second setup, where we compared disease induced by collected versus Allergon pollen.

BAL cell count numbers were significantly increased in mice following treatment with Allergon or urban pollen, compared to mice treated with rural pollen. Furthermore, differences in BAL cell composition showed significantly more inflammatory eosinophils following treatment with urban pollen compared to treatment with rural pollen. However, no clear differences in Ig-levels could be detected in between the different pollen types. Compared to PBS treatment, IgG1 titers were clearly elevated in all mice following pollen-treatment, while IgE titers were only slightly elevated following treatment with rural and urban pollen.

In conclusion, the differences observed following our first approach were validated by the results from our second experiment.

4 Discussion

The aim of this study was to test if environmental factors are able to alter the allergenicity of Ambrosia pollen. Several publications studying the climate change and environmental factors indicate that environmental alteration is contributing to the increase in incidence and prevalence of allergic asthma in the last decades [58, 60, 61, 81]. There is reliable data that the environment is able to alter the timing and quantity of aeroallergen production, and affect emission, dispersion, transport, and deposition of allergens [82, 84, 87-89, 92, 123]. Furthermore, people's health can be affected by direct impact of climate and pollution factors by acting directly on the airways of susceptible individuals, making them more prone to asthma [60, 95-98, 103, 108]. It seems plausible that climate changes and pollution factors may be also able to interact directly with allergens, but few studies addressed this issue so far. Nevertheless, these studies are indicating that climate and environmental factors may alter pollen allergenicity. In addition, recent studies showed that environmental factors are able to alter viability, germination, enzyme expression, and allergen-concentrations of pollen [115, 116, 119]. Moreover, recent research, using in vitro studies with sera from asthmatic patients and single cell type assays, demonstrates that climate and pollution might increase pollen allergenicity [120-122]. Still, data about the direct effect of environmental factors on pollen allergenicity is lacking.

4.1 Development of the allergic asthma protocol

Before we could investigate the possible impact of environmental factors on pollen allergenicity, we had to develop a reliable protocol that induces allergic asthma to Ambrosia pollen in mice. To our knowledge this is the first asthma inducing protocol, using an intranasal administration route, with Ambrosia pollen in mice.

We guided ourselves using allergic asthma-inducing protocols used in the laboratory and other studies to generate and adapt the model to a reliable and reproducible working model for Ambrosia pollen. The response of mice following four-time versus six-time Ambrosia pollen exposure was compared, resulting in the selection of the six-time intranasal treatment with Ambrosia pollen as the model of choice since it caused the most consistent disease onset (Data not shown). Treatment of mice with 6 intranasal applications of adjuvant-free Ambrosia pollen suspended in PBS resulted in clinical features typical for a T_H2 -type pathology and closely resembled allergic asthma [32]. Treatment with all pollen doses (40, 200 and 400 μ g pollen, corresponding approximately to 10, 50, and 100 μ g pollen protein) resulted in elevated

BAL cell counts in all pollen-treated mice, with the lowest counts observed in mice treated with 40µg. All pollen-treated groups developed bronchoalveolar lavage eosinophilia with percentages of eosinophils between 31% and 54%, emphasizing that eosinophils are important mediators of allergic responses and asthma pathogenesis. Various studies have shown that eosinophils represent a predominant feature of late phase asthmatic response and their presence is often correlated with disease severity [32, 124, 125]. Other important pathologic features, such as lung inflammation with eosinophilic infiltrates and mucus hypersecretion, were significantly increased in all pollen-treated mice compared to PBS-treated mice. As expected we did not detect significantly elevated titers of Ambrosia-specific IgG2a, which is a T_H1-induced Ig. While we were able to detect elevated T_H2-vectored Ambrosia-specific IgG1 titers in all pollen treated mice, we were not able to detect Ambrosia-specific IgE in any of the mice. We suspect that the high titer of IgG1 observed in our disease model masks the IgE signal by competing for the available epitopes in our Elisa test. Another explanation might be that IgE, which is critical in human allergic reactions, may not be essential in the development of allergic asthma in mice [32]. Previous work has shown that mice are able to develop allergic airway inflammation in absence of allergen-specific IgE, IL-4 or B-cells [124, 126]. Confirmatively, it has been demonstrated that although IgE plays an important role in the development of airway inflammation, it might not be essential for T-cell activation, eosinophilic airway inflammation, and development of AHR. An IL-5 mediated robust eosinophilic response, independently of IgE or IgE-mediated mast-cell activation, might be sufficient to induce substantial T-cell activation, eosinophilic inflammation, activation in the airways, and development of airway hyperresponsiveness [127].

Intranasal sensitization and challenge with Ambrosia pollen resulted in partly dose-dependent severe and persistent lung and airway inflammation with eosinophilia, mucus hypersecretion, and elevated levels of Ambrosia-specific IgG1.

Both higher pollen dose treated groups (200 and 400µg) showed similar severity in disease manifestations, with no apparent increase when mice were treated with the highest dose, suggesting that maximum disease response was reached at the 200µg dose. In contrast, treatment with 40µg was able to induce disease but resulted in lower airway inflammation and IgG1 titers. Therefore, further experiments were performed using 40µg of pollen per dose, as it seemed to be the most effective dose to detect possible changes in disease severity when pollen was subjected to different environmental stress factors.

Collectively, our results indicate that six-time intranasal administration of pollen induced a T_H2 driven asthmatic response against Ambrosia pollen.

4.2 Treatment of pollen with different environmental factors to determine possible changes of pollen allergenicity

The main objective of this study was to investigate possible effects of climate and pollution factors on the allergenicity of Ambrosia pollen. Having a suitable protocol, we exposed pollen to different climate and pollution factors, including heat, acid rain, copper and nitrate pollution, and assessed disease manifestations after treatment in mice.

We let ourselves be guided by a recent study that investigated the effect of humidity, temperature, acid rain, and copper pollution on pollen viability, germination, and enzyme expression (transglutaminase), indicating that the increase of externalized enzymes may be a mediator of pollen allergenicity [115]. In our study the focus was on pollen treatment with low pH, corresponding to acid rain (pH5, 6) and copper, which are prominent air and soil pollutants.

Although we exposed the pollen to low pH and CuCl₂ solutions before being administered, the results did not indicate any significant change in pollen allergenicity. We observed similar disease severity in all pollen-treated mice and no difference was detected in the allergenicity of treated pollens. We speculate that these findings might be due to the intensity of treatments and time of exposure. One possible explanation may be that concentrations of the pollutants and incubation time may have been insufficient, or further environmental parameters and consequent interactions are needed to induce changes in pollen allergenicity. Another explanation might be that the differences in allergenicity were not detectable by using our asthma animal model. Interestingly, Iorio et al. [115] found differences in pollen viability, germination, and transglutaminase activity after treatment of *M. domestica* and *C. avellana* pollen with the same CuCl₂- and pH-treatments. In our case, similar research scenarios but with Ambrosia pollen did not reveal any effect on pollen allergenicity.

Therefore, we conclude that the used environmental scenarios were insufficient to alter the allergenicity of Ambrosia pollen.

To further investigate potential impact of our tested environmental change scenarios, and to assess if previous results were due to insufficient treatment, we increased CuCl₂ concentrations (10, 100 and 1000µg/L) and lowered the pH for simulated acid rain (pH2, 3 and 4). Moreover, we added two new potential environmental changing models to include heat and nitrate pollution (NH₄NO₃). We mimicked excessive pollution scenarios in an attempt to determine if these new environmental factors, such as CuCl₂, NH₄NO₃ or low pH, have any effects on Ambrosia pollen allergenicity.

In our second round of experiments, we did not detect evident differences in disease manifestations in mice treated with any of the different environmentally treated pollen or untreated pollen. Only in the case of treatment of pollen with a large amount CuCl_2 (1000 μg) there was a significant increase in BAL total cells, compared to pH2-, pH3-, 40°C-, 100 $\mu\text{g/L}$ CuCl_2 - and 10 $\mu\text{g/L}$ NH_4NO_3 -treated pollen. Additionally, eosinophil percentages varied slightly. Although alternations in total cell counts and eosinophil percentages in BAL could indicate trends in changing allergenicity of pollen, the differences in this experiment were only minor. Therefore, we speculate that these effects could be attributable to pollen handling and processing. No other evident difference in disease manifestation was detected in this experiment among all pollen-treated groups. One possible explanation for these findings could be that pollution agents such as CuCl_2 , NH_4NO_3 , and heat or low pH levels do not affect Ambrosia pollen. This theory is supported by the fact that even pollen treated with excessive doses of CuCl_2 , NH_4NO_3 or with very low pH levels, did not undergo evident changes in allergenicity. Several studies indicate that Ambrosia pollen can be affected by climate and pollution factors [116, 119, 120], and since mice treated with excessive amounts of CuCl_2 -pollen showed an evident increase in total BAL cell numbers, we speculate that the pollen allergenicity may be modified to a limited extent, and only under extreme treatments. As disease manifestations in mice receiving untreated company-derived pollen were among the most severe compared to all pollen-treated groups, we concluded that the company pollen (Allergon) elicits an allergic response that cannot be further increased by changes in the environment.

This suggest that company-derived Ambrosia pollen (Allergon) might have been altered during isolation and –processing with an increase in allergenicity that cannot be further affected neither by environmental impact nor by other influences.

4.3 Comparison of pollen allergenicity of company-derived pollen and collected pollen from rural and urban areas

To better investigate the impact of environment on pollen allergenicity, pollen was collected from plants that were growing in either rural or urban (polluted) areas and compared to company-derived pollen (Allergon). First, we wanted to investigate if there are any differences between the allergenicity of pollen derived from rural and urban areas and company-derived pollen. Ambrosia plants collected from both urban areas grew in an

environment where they were constantly exposed to highway pollution, while plants from the rural area grew in an unpolluted environment.

In our first approach we used two different pollen doses (10 μ g and 100 μ g). As expected, application of the low dose resulted in a lower allergic response and all pathogenic features were present, but the response was clearly weaker in all disease instances, compared to the high dose. Interestingly, pollen from the second urban population elicited a slightly stronger allergic response with slightly increased BAL eosinophils and IgG1 titers, compared to the other treated groups. However, we did not detect clear differences between commercial, rural and urban pollen after treatment with the small dose (10 μ g).

In contrast, administration of high doses (100 μ g) displayed some distinct differences in the allergic response between the different pollen types. The results suggest that rural pollen is less allergenic than the other pollen types, as total BAL cells were significantly increased in mice treated with Allergon pollen or both urban pollen compared to PBS, whereas mice treated with rural pollen showed no significant increase. Furthermore, mice treated with rural pollen displayed the lowest percentage of eosinophils with macrophages being the most abundant cell type in BAL fluid. All other pathological features were similar in all groups. One notable difference was that low titers of Ambrosia-specific IgE could be detected in mice that were treated with rural and urban pollen, but not in company pollen-treated mice.

The evident differences in abundance and composition of airway inflammation between rural and both Allergon and urban pollen may indicate that urban and Allergon pollen is broadly similar in allergenicity and is more allergenic than rural pollen.

These findings were supported by subsequent similar findings in our second round of experiments, when comparing Allergon, rural and urban pollen. As before, treatment of mice with company and urban pollen resulted in a significant higher response compared to rural pollen-treated mice. Besides significantly increased total cell counts following treatment with urban or company pollen compared to rural pollen, eosinophilia in BAL was significantly higher in mice treated with urban pollen compared to mice treated with rural pollen. Again, we did not detect differences between all pollen-treated groups in Ambrosia-specific IgG1 titers, but both collected rural and urban pollen was able to induce a very low IgE response.

Both experiments showed increases in airway inflammation and eosinophilia following treatment with urban pollen compared to urban pollen treatment, while no or negligible differences could be detected for the other pathological features. These results suggest that pollen derived from areas that are affected by air pollution might be more allergenic than pollen from rural environments. Pollen collected from plants that were stressed by urban

environment, induced a higher allergic response characterized by increased airway inflammation.

There are various reasons for the increase in Ambrosia pollen allergenicity. Environmental stress factors, and most probably traffic exhaust above all, might affect plants to produce more allergenic pollen or/and interact directly with the pollen itself. A study that compared pollen from rural and urban areas, found quantitative and qualitative differences in allergen patterns, showing a higher whole allergenicity in pollen derived from urban areas [120]. In addition, other studies showed that stress factors such as traffic exhausts could induce an increase or variation in the amount of allergens [118, 119], or facilitate the interaction of the allergen with the immune response by altering antigen processing and presentation [121]. Through alterations in the pollen grains and facilitating the externalization of sub-pollen particles, allergenic proteins or allergy-mediating enzymes may be another possible mechanism that increases the allergenic potential of pollen [115, 122]. Another possible effect of environment on pollen has been revealed by studies in which exhaust particulates, such as diesel exhaust particulates, were shown to be able to bind proteins and act as adjuvants to allergens, increasing the allergic response [108, 109].

Our results support some of these findings and validate that stressful environmental factors can impact pollen, increasing the allergenic potential. The mechanisms involved in the increase of pollen allergenicity concerning our study remain undetermined. It is still unclear if the environmental stress is targeting the pollen directly, or if the plant is induced to produce pollen that is more allergenic.

In conclusion, we successfully developed an *in vivo* model to investigate Ambrosia pollen allergic asthma in mice. This model was used to determine the impact of environmental factors on Ambrosia pollen allergenicity. Although we mimicked a wide range and various doses of environmental factors, including heat, acid rain, copper and ammonium nitrate pollution, we were not able to detect significant differences in disease severity when using processed pollen. We conclude that the company-derived pollen (Allergon) might have been altered during production and processing in such a way that further alterations in pollen allergenicity may not be possible. Subsequently, we collected pollen from rural and urban areas and compared the allergenicity with company-derived pollen. Increased pathological outcomes in mice treated with urban pollen indicated that pollen from plants derived from stressful environments might be more allergenic than pollen from rural areas. Environmental stress factors, in our case most probably traffic pollution, might directly impact plants to

produce a more allergenic pollen or/and may interact directly with the pollen itself. Moreover, treatment with Allergon pollen and urban pollen resulted in similar disease severity, supporting our assumption that Allergon pollen allergenicity is being altered during processing. Our results suggest that company-derived pollen has been altered during production and processing, and collected rural pollen should be used in further experiments to investigate possible effects of environmental stress factors on pollen allergenicity.

Urban areas are undergoing a permanent expansion, and this correlates with the related pollution affecting plants and pollen. Based on these results from our study, there is a strong indication that the observed aggravating effects of urban pollution on pollen allergenicity might be a major contributor to the global rise in incidence and prevalence of allergic asthma.

5 Summary

Allergic asthma is a chronic immune-mediated inflammatory disease of the respiratory tract, characterized by airway dysfunction induced by a hypersensitivity reaction against allergens. Several publications studying the climate change and environmental factors indicate that environmental alteration is contributing to the increase in incidence and prevalence of allergic asthma over the last decades. It has been suggested that environmental factors, such as extreme temperatures, changes in humidity, low pH, and pollution may interact directly with allergens, altering allergenicity.

We developed an allergic asthma model for disease induction in BALB/c mice with the highly allergenic pollen of *Ambrosia artemisiifolia* to investigate possible differences in allergenicity of pollen incubated under several stress conditions. Although we mimicked a wide range and various doses of environmental factors, including heat, acid rain, and copper and ammonium nitrate pollution, we were not able to detect significant differences in disease severity when using processed pollen. We hypothesized that the purchased untreated pollen may have been modified during processing and could not be altered further under the conditions used in our study. To address this hypothesis, we planned to use locally collected *Ambrosia* pollen to detect possible changes in allergenicity following treatment with environmental stress conditions. Therefore, we collected pollen from rural and urban areas and compared the allergenicity with company-derived pollen. Rural pollen was harvested from an area in the countryside unexposed to air pollution, whilst urban pollen was collected from the vicinity of a highly motorized roadway where plants were constantly exposed to exhaust fumes. Increased pathological outcomes in mice treated with urban pollen indicated that pollen from plants derived from high stress environments might be more allergenic than pollen from rural areas. Environmental stress factors, in our case most probably traffic pollution, might directly impact plants to produce a more allergenic pollen or/and may interact directly with the pollen itself. Moreover, treatment with Allergon pollen and urban pollen resulted in similar disease severity, supporting our assumption that Allergon pollen allergenicity is being altered during processing.

Our results suggest that company-derived pollen has been altered during production and processing, and collected rural pollen should be used in further experiments to investigate possible effects of environmental stress factors on pollen allergenicity. Furthermore, we suggest that the observed aggravating effects of urban pollution on pollen allergenicity might be a major contributor to the global rise in incidence and prevalence of allergic asthma.

6 Zusammenfassung

Allergisches Asthma ist eine chronische immun-vermittelte Entzündungserkrankung der Atemwege, die durch eine Überempfindlichkeit gegen Allergene hervorgerufen wird. Mehrere auf den Klimawandel und auf Umwelt-Faktoren bezogene wissenschaftliche Veröffentlichungen weisen darauf hin, dass Umwelt-Veränderungen in den letzten Jahrzehnten dazu beigetragen haben, das Vorkommen von allergischem Asthma zu erhöhen. Umweltfaktoren, wie extreme Temperaturen, Veränderungen in der Luftfeuchtigkeit, niedrige pH-Werte und Schadstoffe stehen im Verdacht u.a. direkt mit Allergenen wechselzuwirken und dadurch die Allergenität zu verändern.

Um mögliche Unterschiede in der Allergenität von Pollen, unter verschiedenen Stress-Bedingungen zu untersuchen, entwickelten wir ein Protokoll um allergisches Asthma mit dem hoch-allergenem Pollen von *Artemisia artemisiifolia* in BALB/c Mäusen zu induzieren. Obwohl wir ein breites Spektrum an Umweltfaktoren und verschiedene Konzentrationen von diesen imitierten, einschließlich Hitze, sauren Regen, Kupfer- und Ammoniumnitrat-Verschmutzung, konnten wir keine signifikanten Unterschiede im Krankheitsbild finden. Wir vermuteten, dass der gekaufte Pollen während der industriellen Verarbeitung so modifiziert worden war, dass die Allergenität durch Umweltfaktoren nicht mehr weiter veränderbar war. Um dennoch mögliche Auswirkungen von Umwelt-Stressbedingungen auf die Allergenität von Pollen zu untersuchen, entschieden wir lokal gesammelten Pollen zu verwenden. Wir sammelten Pollen aus ländlichen und städtischen Gebieten, und verglichen die Allergenität mit der von gekauftem Pollen. Während der Pollen aus dem ländlichen Gebiet keiner oder wenig Schadstoffbelastung ausgesetzt war, wurde der Pollen aus der städtischen Umgebung in unmittelbarer Nähe einer stark frequentierten Autobahn gesammelt und war somit einer massiven Schadstoffbelastung des Verkehrs ausgesetzt. Mäuse, die mit städtischem Pollen behandelt wurden zeigten ein ausgeprägteres Krankheitsbild als Mäuse, die mit Pollen aus dem ländlichen Gebiet behandelt wurden. Jene Mäuse, die mit dem gekauften Pollen behandelt wurden, wiesen ähnlich starke Krankheitssymptome auf wie jene nach Behandlung mit städtischem Pollen.

Wir schließen daraus, dass der gekaufte Pollen während der Herstellung verändert wurde und empfehlen deshalb Pollen aus wenig belasteten ländlichen Gebieten zu verwenden, um mögliche Auswirkungen von Umwelt-Stressfaktoren auf die Allergenität von Pollen zu untersuchen. Außerdem deuten unsere Ergebnisse darauf hin, dass städtische Schadstoffbelastung sich erschwerend auf die Allergenität von Pollen auswirkt, und zum globalen Anstieg von allergischem Asthma beiträgt.

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Curriculum vitae

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