THE INFLUENCE OF INFECTIONS ON THE DEVELOPMENT OF ALLERGIC DISORDERS

Ph.D. Thesis

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List of abbreviations

AHR	Airway Hyperreactivity
APC	Antigen Presenting Cell
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
BCG,	*Bacillus Calmette-Guerin*
BCG-CWS	Cell Wall Skeleton of BCG
BHR	Bronchial Hyperresponsiveness
CCR	Chemokine Receptor
CFU	Colony Forming Unit
COPD	Chronic Obstructive Pulmonary Disesase
CpG-ODN	Citozin-poli-Guanidin-Oligodinicleotids
DC	Dendritic Cell
DNA	Dezoxi-Nuclein Acid
DTH	Delayed Type Hypersensitivity
ECRHS	European Community Respiratory Health Study
ELISA	Enzyme Linked Immuno Assay
Flu	Influenza virus
Flu-Ag	Influenza virus Antigen
GINA	Global Initiative for Asthma
HK -BCG	Heat-Killed *Bacillus Calmette-Guerin*
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
ISAAC	International Study of Asthma and Allergies in Childhood
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MLN	Mediastinal Lymph Nodes
Nippo	*Nippostrongylus brasiliensis*
NK	Natural Killer
OVA	Ovalbumin
PBS	Phosphate-Buffered Saline
PIT	Peptide-Based Immunotherapy
PPD	Purified Protein Derivative of *Mycobacterium tuberculosis*
RANTES	Regulation upon Activation Novel T cell Expressed and presumably Secreted
RSV	Respiratory Syntitial Virus
SIT	Specific Immunotherapy
STAT	Signal Transducer and Activator of Transcription
TGf-β	Tumor Growth Factor-beta
Th	T helper
TNF-α	Tumor Necrosis Factor-alpha
Tr	Regulatory T cell

1 Preface

This thesis is based on the following articles, referred to in the text by their Roman numerals

I. Major, T., Wohlleben, G., Reibetanz, B. & Erb, K.J. Application of heat killed mycobacterium bovis-BCG into the lung inhibits the development of allergen-induced Th2 responses. *Vaccine* 2002; 20: 1532-40.

II. Grunewald, S. M., Hahn, C., Wohlleben, G., Teufel, M., Major, T., Moll, H., Brocker, E. B. Erb, K. J. Infection with influenza a virus leads to flu antigen-induced cutaneous anaphylaxis in mice. J Invest Dermatol 2002;4: 645-51

III. Major, T., Szilvasy, B., Wohlleben, G., Erb, KJ. Heat-killed Mycobacterium bovis- Bacillus Calmette-Guerin prevents experimental respiratory tract eosinophilia in mice. Orv Hetil. 2002;143(22):1361-6.

2 Introduction

2.1 Definition of asthma

Asthma is approximately coeval with mankind. The earliest relics originate from the ancient Chinese culture. The legend of the „Three Emperors" date back to approximately 2700 B.C. They were not really emperors, rather famous scientists, who not only diagnosed, but treated allergic diseases. The „Ebers Papyruses" (3000-1200 B.C.) witness the knowledge of Egyptian asthma remedy. The term asthma, still used today, comes from Hippocrates (460-377 B.C.), the founder of Greek and classical medicine [1].

Hippocrates:
Father of Medicine

Figure 1.

"Asthma is a word of Greek origin that means to breathe hard or to pant. One of the first people to write about asthma was Hippocrates, the renowned *Father of Medicine* (**Figure 1**). Hippocrates has been credited with establishing medicine as a science, an art, and a profession of great dignity. His collection of essays and texts form the basis of Western medicine. His name entered the domain of popular culture through its association with the "Hippocratic Oath," a brief text that was once honoured as the nucleus of Western medical ethics. Though this pioneer of science was alive over two thousand years ago, Hippocrates was able to recognize the spasmodic nature of asthma and believed its onset to be caused by moisture, occupation, and climate. He suspected that asthma was comparable to epilepsy and had "its own nature" arising from external causes."[2]

From Roman times the names of Celsus, Aretius of Kappadocia, and Galenus should be mentioned. Asthma was also known to the ancient jewish medicine. The *Gemara*, part of the Talmud explaining the laws, describes asthma among other diseases. The ancient Indian culture should also be mentioned, where writings about asthma can also be found. Sir J. Floyer (1649-1734) the famous phisician medieval from the medieval ages, describes the treatment of asthma in details in his book: „A Treatise of the Asthma", London, Printed for Richard Wilkin, 1698.). Sir Thomas Willis, another famous British researcher recognized the role of the nervous system in the the pathogenesis of asthma. Moreover he described the basic three asthma sympthoms: mucus-plugging, epithelial edema and smooth muscle spasm, wich was than named „Virchow trias" after Virchow. [1]

Although physicians have not been able to agree on a universal definition of asthma since 1992 [3], most patients with the disease know that they have asthma and can describe it. The difficulty with the definition reflects our poor understanding of both the disease and the factors that control the airway lumen in health. At the Ciba Symposium in 1952 [4], asthma was

defined as "the presence of widespread narrowing of the airways which alters in severity either spontaneously or in response to treatment." In 1962, the American Thoracic Society [5] modified the wording to include the phrase "characterized by increased responsiveness". This definition, which was to remain the standard until 1992, was unsatisfactory because it ignored the issue of whether a single definable disease with its own unique pathogenesis and pathology exists within the syndrome of diseases labelled clinically as asthma.

Over 100 years ago, the basic abnormality in people with asthma was recognized as an increased airway abnormality [6]. In 1946, this irritability was defined in terms of changes in the vital capacity in the response to inhaled histamine [7] and in 1958, the fundamental abnormality in patients with asthma was recognized as an excitability of the smooth muscle [8]. It is only in the last two decades that the importance of bronchial hyper-responsiveness (BHR) as the underlying feature of asthma has become widely recognized [9]. However, defining asthma in terms of BHR alone is inadequate because increased responses to provoking factors such as histamine and methacholine are also found in some subjects without symptoms [10] and in many subjects with chronic obstructive pulmonary disease (COPD) who do not have asthma [11]. In addition, spontaneous or induced attacks of asthma can result from exposure to ragweed pollen in people who do not have hyper-responsiveness to methacholine [12]. Moreover, some non-allergic individuals without hyper-responsiveness but with symptoms of asthma have been described [13].

The current definition of asthma was introduced at the International Consensus Report [14] in 1992, placing airway inflammation into focus, and modified in the GINA- Global Initiative for Asthma Guidelines in 1997[15].

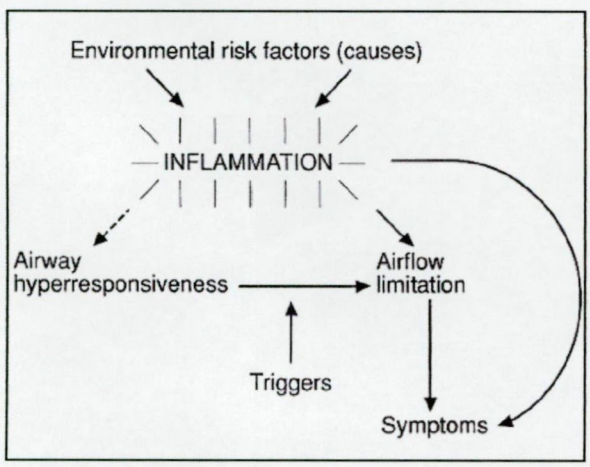

Figure 2. *The « modern view » of asthma*

Asthma may be defined based on pathology and its functional consequences. A greater understanding of asthma management has been achieved by accepting the persistence of the chronic inflammatory response, with variations in the magnitude of the inflammation reflecting the clinical activity of asthma (**Figure 2**). Because there are no well-validated non-invasive measurements of airway inflammation

in asthma, the clinician and epidemiologist have had to rely on surrogate indices. Based on the functional consequences of airway inflammation, an operational description of asthma is that: "*Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils, and T lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or in the early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli.*" [16]

2.2 Epidemiology

Asthma is a problem worldwide and the social burden and costs to both public and private health care systems are substantial. There is strong evidence that asthma prevalence has been increasing in many countries, but as yet there are insufficient data to determine the likely causes of this increase or of the described variations in and between populations. The existing data on asthma prevalence are derived mainly from populations in developed countries. There are insufficient data on the severity of the disease in different populations and on the impact of asthma management guidelines.

Despite hundreds of reports on the prevalence and mortality of asthma in widely differing populations, the lack of precise definitions of asthma makes reliable comparison of reported prevalence from different parts of the world problematic. However, the recent application of standardized methods to measure the prevalence of asthma and wheezing illness in children [17] and adults [18] has aided in making such regional and international comparisons. Some data from phase 2 of the International Study of Asthma and Allergies in Childhood (ISAAC) permit between-population comparison of airway hyper-responsiveness, lung function, peak flow variability, and atopy in children [19,20]. The European Community Respiratory Health Study (ECRHS) [21] enabled between-population comparisons of airway hyper-responsiveness, atopy, and symptoms of asthma in adults, but so far these three aspects of asthma have not been correlated. Thus, because no epidemiological definition of asthma is emerging from current data, important components of epidemiological studies for asthma continue to include questionnaires, tests of airway hyper-responsiveness, and documentation of putative etiologic factors including atopic status.

2.2.1 Prevalence of asthma

The prevalence of asthma symptoms in children varies from 0 to 30 percent in different populations.

There are large differences in prevalence among populations, with the highest prevalence of asthma found in England and Australia. Data are insufficient to determine whether the differences between populations are the consequence of responses to the environment, to industrialization, or to different allergen loads. Although there is some evidence that asthma is less prevalent in children with high levels of parasitic infections [22], there have been no systematic studies relating parasitic infection to asthma where adjustment was made for other environmental factors[23].

There are relatively few data on adults. Asthma occurs in all races. Although it is clear that genetic factors are of major importance as predisposing factors in the development of atopy, and probably asthma, evidence suggests that environmental rather than racial factors are important in the onset and persistence of asthma (**Figure 3.**) [24].

Even though good epidemiological evidence is difficult to obtain, studies clearly suggest a true increase in asthma prevalence worldwide. The prevalence of asthma has increased in the past two to three decades in both children and young adults [17,22].

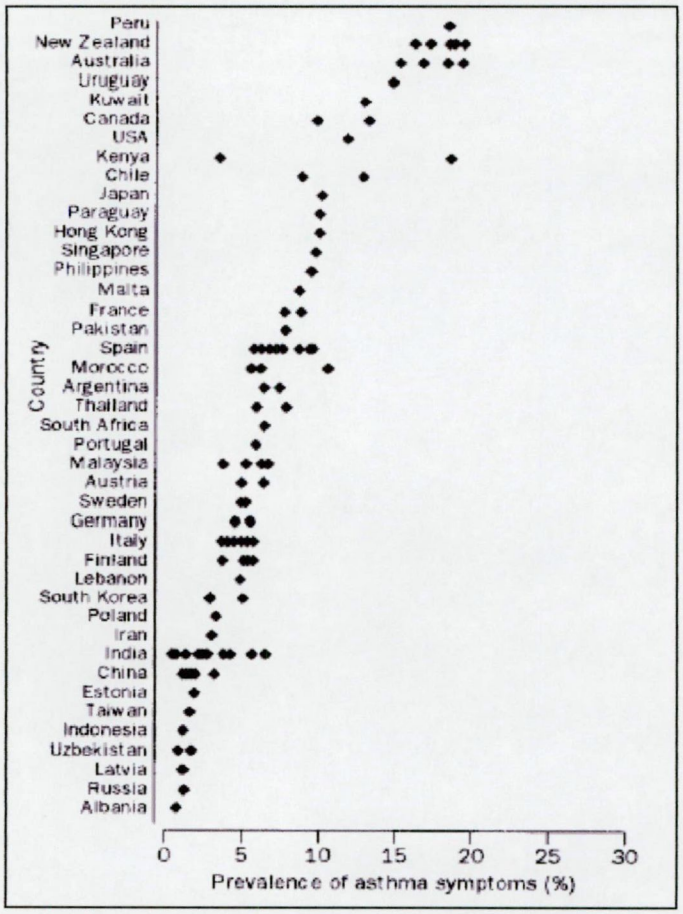

Figure 3. *From the ISAAC study, shows the prevalence of wheezing in the last 12 months–documented by written questionnaires–among children 13 to 14 years old in a number of populations. The data show a wide range in the prevalence of wheezing in different populations*

In Hungary, the prevalence of asthma is lower than in the above mentioned countries, but it shows an increasing tendency. By the year 2002 the registered number of asthma patients has reached 1.5% of the whole population with

18000 new cases encountered (**Figure 4**). These results — in comparison with the international prevalence data—are much lower than the real number of asthmatics. It can be

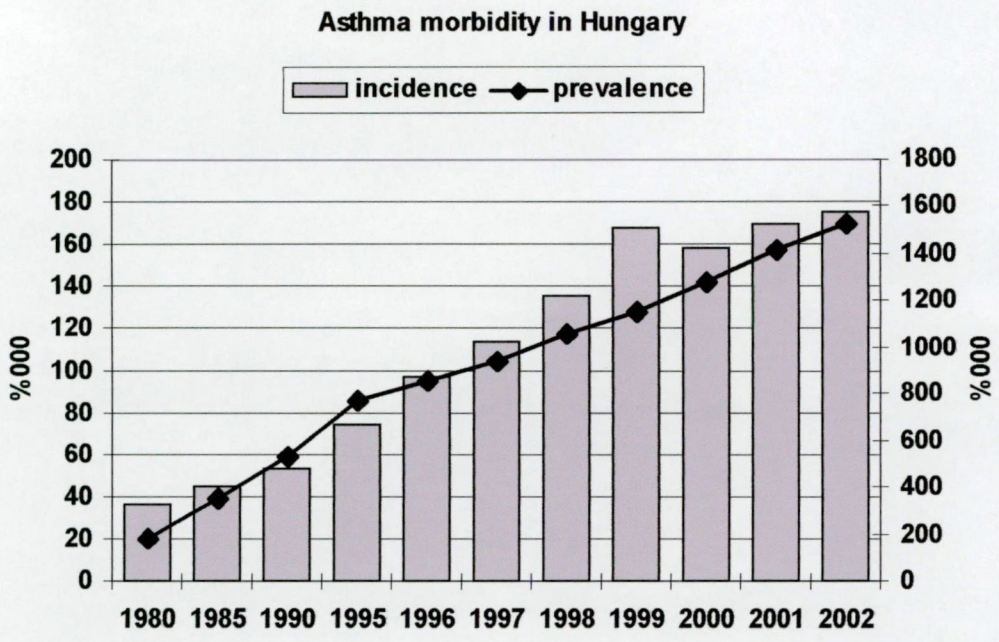

Figure 4. *Asthma incidence and prevalence in Hungary.*

Taken by permission from the Annual Report of the Hungarian Medical Care Centers in Respiratory Medicine. Issued by: National Koranyi Institute for TBC and Respiratory Medicine. Budapest, Hungary

assumed, that mostly the intermediate persistent and severe cases are registered in Hungary. Therefore, there are no valid prevalence data among the adults in Hungary. However, the prevalence of asthma in children and young adults is closer to the international data and varies between 2-6% [25].

The relationship between atopy and asthma is known for decades. Moreover, atopy is the strongest risk factor in the development of asthma.

2.3 Genetic predisposition

It is known that atopic diseases show a clear familial aggregation. The genetic control of allergy is multigenic [26] and diverse etiological processes causing partially overlapping clinical phenotypes. The genetic background of atopy was first pointed out by D. Marsh et al. more than two decades ago. They have found that the association between positive skin prick test reactions to a ragweed pollen antigen and class II haplotype HLA-DR2. With these results

they proved the linkage between specific IgE responsiveness and the HLA gene complex. [27,28].

The rapid development of molecular methods made it possible to describe primary amino acid sequence of many allergens, as well as the structure of HLA class II molecules. These findings led to the analysis of allergen/class II molecular interactions in detail. Moreover, the observation of T cell dependent specific IgE production called the scientists' attention to the analysis of the HLA restriction specificity of T cell responses to allergens. It has been shown that HLA class II genes and T cell receptor genes are controlling the specific IgE response. [29] Candidate genes were detected in 5q 31.1 – 33.3, which is the region encoding numerous Th2 cytokines and β2 adrenergic receptors [30] and in chr 11q 12-13, which encodes the β chain of high affinity IgE receptor [31].

Despite the large quantity of research data increasing our knowledge on structural/functional relationship at the molecular level, association between HLA and appearance of symptoms has had a minimal impact on treatment of allergic disorders. In conclusion, it is required to study the pattern of inheritance in a larger number of families, especially in identical twins, as well as to answer the question whether the interaction between atopy genes, presumably controlling polyclonal processes in immune response and HLA genes involved in immune recognition.

2.4 Pathogenesis of asthma

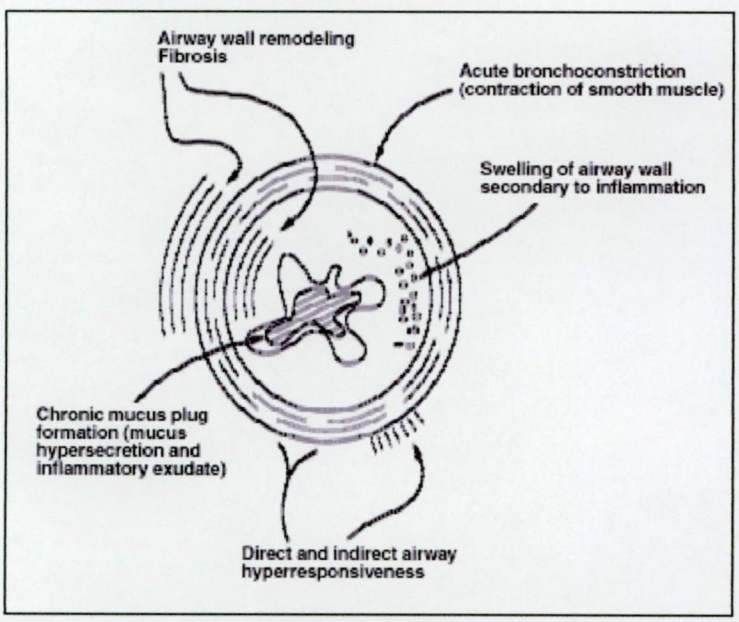

Figure 5. *Factors that contribute to airflow limitation in asthma*

Clinically, in patients experiencing an asthmatic attack, the lung becomes over-inflated and both the large and small airways fill with mucus plugs containing proteins, desquamated epithelial cells, and inflammatory cells. In histological sections, extensively infiltrated airway walls with eosinophils and lymphocytes and devastated ciliated epithelial membrane are

accompanied by excessive interstitial oedema as a result of microvascular leakage **(Figure 5)** [32,33]. Chronic injury results in smooth muscle hypertrophy, new vessel formation, increased numbers of goblet cells, and interstitial collagen deposition beneath the epithelium can be identified, which may lead to remodelling [34].

There is a considerable amount of evidence suggesting that mast cells and eosinophils play a key role in the inflammatory response by secreting a wide range of preformed and newly synthesized mediators that act on the airway effector cells directly and indirectly through neural mechanisms [35].

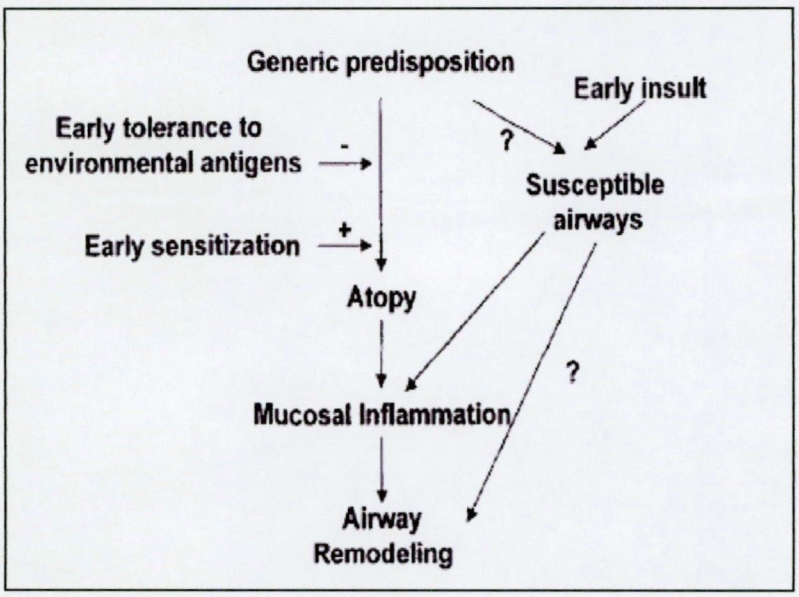

Figure 6. *Pathogenesis of asthma. Inflammation is not sufficient to cause asthma, but the susceptibility of the airway structures to damage by chronic inflammation may be critical. The origin of the susceptibility is unknown, but it could be inherited or it could result from injury. From:* ***www.ginasthma.com***

With the development of immunological and molecular biological assays in asthma research, T lymphocytes have been identified as key elements in controlling the inflammatory response through the release of multifunctional cytokines [36]. The allergic immune response in asthma involves Th2 cells. The generation of cytokines produced by the constitutional cells of the airways, such as fibroblasts, endothelial cells, and epithelial cells, are increasingly considered to be essential in the regulation of the inflammatory response [37]. Moreover, immune-competent cells in the airway epithelium, also called as BALT (Bronchial Associated Lymphoid Tissue) plays an active role in the regulation of allergic immune response as a first line defence system [38].

In addition to airway smooth muscle contraction, interstitial oedema, neuronal activation, and stimulation of mucus-secreting goblet cells, numerous other factors (i.e., cytokines, chemokines, and growth factors) are released that have the potential to attract other inflammatory cells and facilitate the inflammatory process inducing a vicious circle which

leads to structural damage of the bronchial tissue. The ciliated epithelium is one major target of this attack, which becomes peeled and transforms into a single layer of cuboid cells [39]. Moreover, subepithelial fibroblasts may proliferate, thus giving rise to collagen deposition in the basement membrane [40].

Chemokines, such as RANTES and eotaxin, are particularly important in the recruitment of inflammatory cells to the airways. The release of mediators and the regulation of the inflammatory process in asthma are complex, redundant, and self-perpetuating (**Figure 6**).

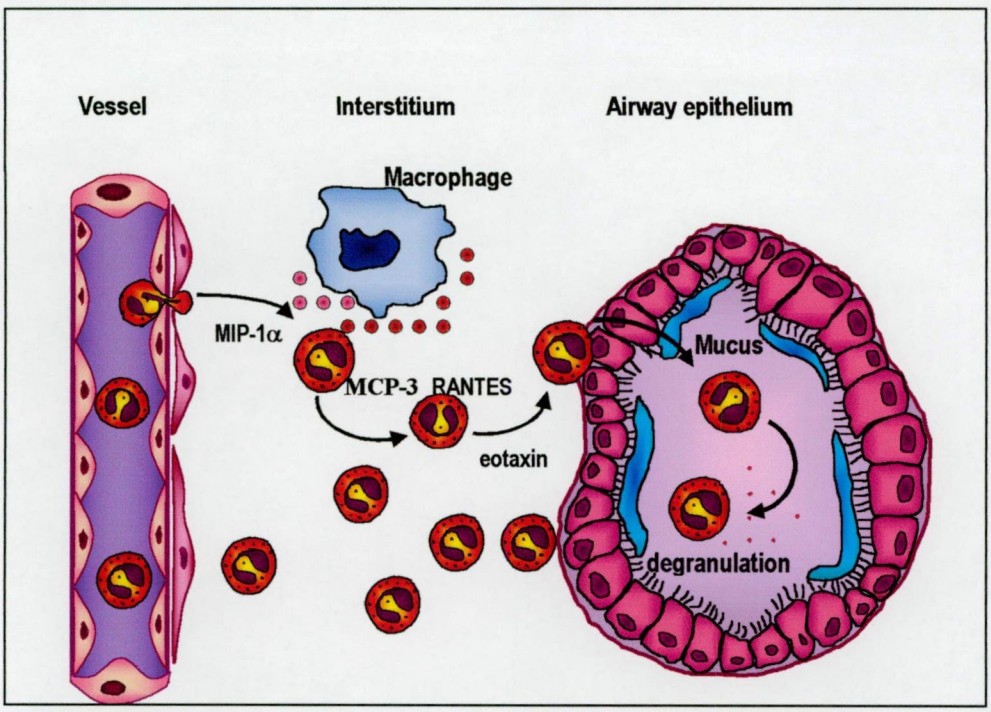

Figure 7. *Chemokine-induced migration of eosinophils in lungs. Eosinophil migration from the vascular supply to the airway is probably dependent of sequential chemokine gradients, which allow the cells to extravasate through the various compartments of the lung.*

According to a widely supported hypothesis, airway inflammation alone is not sufficient for the disease to develop. Airway eosinophilia is not strongly associated with bronchial hyper-reactivity in either adults or children. For asthma to develop and persist, the airways must not only be inflamed, but the mucosa must also be susceptible to injury from chronic inflammation. It is the interaction between the inflammation and the airway structures that lead to the full asthma syndrome[41] (**Figure 7**).

2.4.1 Early and late phase allergic reaction in asthma

After the first exposition to allergen, the cells present in asthmatic airways are already in sensitized condition, showing a "pro-inflammatory" phenotype. The immune response induced by further exposures can be divided into two distinct phases. The early phase occurs quite rapidly due to these further exposures to allergen, when allergens penetrate through the mucosa. They are then taken up by antigen-presenting cells (APCs), which present them to Th2 lymphocytes. The dendritic cells (DCs) localised in the airway epithelium, react faster compared to the alveolar macrophages, because they bind the antigens without fagocytosis. The Th2 lymphocytes produce IL-4 and IL-13, which activates the antigen-specific B lymphocytes causing specific IgE antibody production. These specific IgE then bind to the high affinity IgE receptors (FcεRI) of the basophil leukocytes and mast cells. The first allergen-exposition causes only the sensitivity of the effector cells. Further expositions, when the specific allergen reacts with sensitized basophil and mast cells cause the activation of these cells by the cross-linking of the FcεRI molecules. This induces degranulation of the effector cells, which causes mediator release: i) preformed mediators, such as histamine, and ii) many newly synthetized mediators, such as leukotriens, prostaglandins, platelet-activating factor (PAF), and various cytokines. These mediators are responsible for the main characteristic features of the asthmatic attack: smooth-muscle spasm, interstitial oedema, increased vascular permeability and subsequent changes in airway function. Prostaglandins and leukotriens, as *de novo* mediators are formed after degranulation of the cell membrane from arachidonic acid. Among the sulfopeptid leukotriens, LTC4 and LTD4 are responsible for the smooth-muscle spasm and increased vascular permeability, while LTB4, as a potent chemoattractant, facilitates the penetration of effector cells into the bronchial tissue.

Mediators released during the early phase in combination with allergen-specific immune responses cause subsequent progression into the late-phase response. The late-phase response is often more severe and is characterized by the accumulation of mononuclear cells (monocytes and lymphocytes) as well as eosinophils. The cell populations that accumulate during the late-phase response are associated with prolonged airway dysfunction and damage. Eosinophils produce numerous mediators, such as eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), which damage the bronchial epithelium and/or induce direct degranulation of the mast cells. These harmful

effects result in subepithelial fibrosis of the asthmatic bronchial wall and the loss of flexibility of the lung parenchyma, leading to structural remodelling [38].

2.5 Risk factors

2.5.1 Air pollution

The role of air pollution in the development of asthma and allergy was studied by comparing the prevalence of respiratory disorders in school children living in two German cities: the eastern city of Leipzig, with its heavy industrial pollution, and the western city of Munich, with its heavy automobile traffic. Asthma and allergy were significantly more prevalent in Munich, while bronchitis was more prevalent in Leipzig [42]. This difference was related to, among various other factors, the type of air pollution: in Munich, vehicle emissions (nitrogen dioxide and respirable particulate matter) predominated, whereas in Leipzig the air pollution contained high concentrations of sulphur dioxide from high-sulphur coal burned for heating and energy production. Subsequent studies [42,43] demonstrated that the prevalence of hay fever in Leipzig schoolchildren increased from 2 - 3 percent in 1991-92 to 5.1 percent in 1995-96 (i.e., 6 to 7 years after the reunification of Germany), while the prevalence of all IgE-mediated sensitizations increased from 19.3 percent in 1991-92 to 26.7 percent in 1995-96. Thus, the difference in prevalence of allergic disorders previously seen between eastern and western Germany is rapidly diminishing as motor vehicle traffic and the consequent forms of air pollution increase in the former East Germany[20,44].

2.5.2 The hygiene hypothesis

In 1989, Strachan proposed a novel but speculative explanation for the apparent rise in the prevalence of allergic diseases.[45] Colloquially named the "hygiene hypothesis", it states: *"These observations . . . could be explained if allergic diseases were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally . . . Over the past century declining family size, improved household amenities and higher standards of personal cleanliness have reduced opportunities for cross- infection in young families. This may have resulted in more widespread clinical expression of atopic disease."*
At first this hypothesis was received with skepticism because the prevailing immunological thinking considered infection to be a potential trigger of allergic sensitization rather than a

protective influence [46]. However, during the early 1990s, a plausible mechanism arose from the distinction of Th1 and Th2 lymphocyte populations in laboratory animals and the recognition that "natural immunity" to bacterial and viral infections induces a Th1 pattern of cytokine release, potentially suppressing the Th2 immune responses involved in IgE-mediated allergy. Although the Th1/Th2 paradigm may not be as clear in humans as it first appeared in rodents, the "hygiene hypothesis" has remained a subject of interest to both immunologists and epidemiologists throughout the 1990s[47,48].

The recent epidemic of atopic disease and asthma may have occurred as a consequence of a decline in certain childhood infections or a more general lack of exposure to a broad range of infectious agents in the first years of life. This idea is supported by studies of children in environments that would be expected to lead to increases in viral infections. Ball *et al.* investigated 1035 children as part of the Tucson Children's Respiratory Study [49]. Children with older siblings at home or who attended day care and, therefore, were more likely to be exposed to respiratory viral infections, were more likely to have frequent wheezing (more than 3 episodes in the preceding year) at age 2 years, but were less likely to have frequent wheezing from ages 6-13 years (**Figure 8**). Such children have a reduced risk of atopic sensitization and asthma at school age [49].

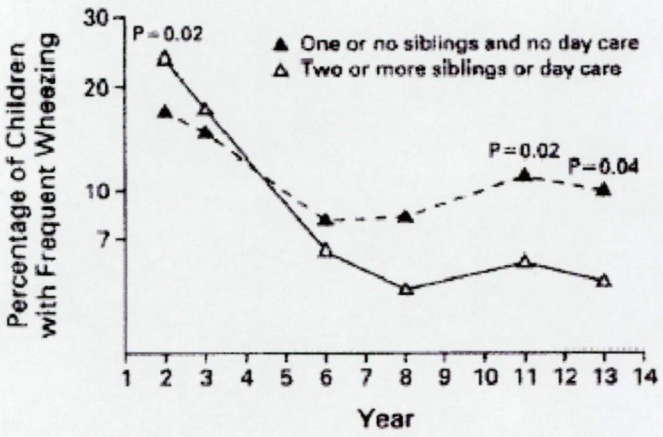

Figure 8. *Prevalence of frequent wheezing among children who had two or more older siblings or who attended day care during the first 6 months of life and among children with less exposure to other children. Children with less exposure to other children were those who had one or no older siblings and who did not attend day care during the first 6 months of life. P values are for the comparisons between the two groups of children. From: www.ginasthma.com*

Improvement in hygiene and reduced recirculation of common infections is reported to be strongly associated with the increasing prevalence of atopy and atopic diseases in Western countries [50]. Respiratory allergy seems to be less frequent in people heavily exposed to orofecal and foodborne microbes. Hygiene and a westernized, semi-sterile

diet may facilitate atopy by influencing the overall pattern of bacteria that stimulate the gut-associated lymphoid tissue (GALT), thus contributing to the epidemic of allergic asthma and rhinitis in developed countries [50]. The most consistent evidence of an inverse relationship between infection and allergy is known from studies of hepatitis A [51].

A possible link between mycobacterial infection and reduced risk of allergy was suggested by a study of 867 Japanese children who underwent routine tuberculin tests prior to *Mycobacterium bovis* BCG vaccination at ages 6 and 12 years. An inverse relationship was observed between delayed hypersensitivity to tuberculin at the age of 12 and both total and allergen-specific serum IgE levels at the same age [52]. The authors interpreted this as evidence that prior infection with tuberculosis or environmental mycobacteria might protect against the development of allergy. However, the same data may simply suggest that atopic/asthmatic subjects have a less prominent tuberculin response. Conflicting data have been reported on the relationship between BCG vaccination and the development of atopy and asthma [53]. The role of other vaccinations, including those for measles and pertussis[54,55], has been questioned. Another factor that confers protection against the development of atopy and asthma is growing up on a farm [56]. Living conditions of farming families differ in many respects from those of other families: larger family size, more pets, frequent heating with wood or coal, less maternal smoking, more dampness, and different dietary habits. None of these factors, however, explains the strong inverse association between atopy and growing up on a farm. Instead, contact with livestock and poultry has been found to explain much of the relationship between farming and reduced prevalence of atopy [57].

3 Aims of the studies

3.1 To investigate the effects of Th1 immune response on the development of allergen-induced airway eosinophilia (I.)

The focus of our interest was to investigate the effects of Th1 immune response on the development of allergen-induced airway eosinophilia. To generate a strong Th1 immune response, live *Bacillus Calmette-Guerin* (BCG), heat-killed *Bacillus Calmette-Guerin* (HK - BCG), and Purified Protein Derivative of *Mycobacterium tuberculosis* (PPD) were used prior to the OVA immunization.

The suppressive effect of BCG on OVA-induced airway eosinophilia was reported earlier by the group. The main objective of the present study series was to investigate whether the bacterium has to be alive to preserve this property.

3.2 To evaluate the effects of a pre-existing helminth infection inducing Th2 immune response on allergic type Th2 reaction

An additional objective of our work was to evaluate the effects of a pre-existing helminth infection inducing Th2 immune response on allergic type Th2 reaction. For this reason a series of studies were performed where mice were infected with helminth and thereafter immunized with OVA according to the protocol described above. To generate a strong Th2 immune response in mice, a metazoic worm species, *Nippostrongylus brasiliensis*, which is a ubiquitous pathogen among rodents, was used

3.3 To investigate the role of Major Histocompatibilitiy Complex class I molecules on allergic inflammation

Exploring the details of antigen presentation in allergic reactions, the role of Major Histocompatibility Complex (MHC) class I cell surface molecules became the focus of our interest. The aim of this work was to identify whether Th2 immune response can be generated by OVA immunization without the presence of MHC class I molecules. To resolve this issue, a study was performed in which MHC class I deficient and control mice were treated under the same OVA-immunization protocol.

3.4 To explore the effects of a viral infection on a pre-existing allergen immunization (II.)

According to accumulating epidemiological data and clinical observations, the role of Th1 response-inducing infections in the development of allergic disorders is controversial. As discussed previously, these agents may be responsible not only for suppressing the Th2 responses in an immature immune system, but also for triggering exacerbations of allergic symptoms. This is especially true of respiratory viruses, such as influenza or RSV. To explore the effects of an airway viral infection on a pre-existing allergen immunization, BALB/C mice were challenged with OVA intranasally at one day intervals and subsequently infected with influenza A virus.

Materials and methods

3.5 Ovalbumin immunization (I, III)

For modelling the development of allergic reaction, intraperitoneal and finally intranasal ovalbumin (OVA) was administered in C57Bl/6 mice resulting in a strong influx of eosinophils into the airways (see the 1st attached publication in the appendices: "materials and methods" section). This experimental animal model was implemented to simulate this key step of allergen-induced immune reactions (Figure 14). When OVA are injected intraperitoneally, allergic sensitization occurs where the naïve T cell clones develop into committed Th2 cells. As a consequence of intranasal OVA boost, a profound allergic, Th2–type immune response occurs in the airways involving the recruitment of inflammatory cells including eosinophils. This model was based on the results of the kinetic studies: mice were sacrificed six days after OVA intranasal challenge, blood samples were taken and broncho-alveolar lavage (BAL) was performed. Lavage cell smears were made by cytospin and the cell counts were determined under light microscopy. Cytokine levels were determined in the culture supernatants from in vitro re-stimulated T cells from the draining mediastinal lymph nodes (MLN) by ELISA. This model was used in a series of experiments where the effects of various infectious agents on the development of allergic inflammation were tested.

To establish an animal model for an ongoing allergen-specific Th2 response where recurrent antigen exposure occurs, we applied OVA intranasally once weekly for six weeks [58,59]. This model was used in our influenza virus experiments where the effects of Th1 immune response inducing viral infections were observed on allergen-specific Th2 response.

3.6 Treatment of mice with BCG, HK-BCG or PPD (I, III)

To induce Th1 immune response prior to the development of OVA-induced Th2 response, mice were treated with BCG, heat-killed BCG HK-BCG, (Toxoplasma in some unpublished experiments) or PPD.

Mice were anaesthetized and intranasally treated with different amounts of live and HK-BCG or with 40 μg of PPD at 8, 4 or 1 week before the intranasal OVA application. BCG was heat killed by incubating the mycobacteria two times for 1h at 80 °C in a water bath. The viability of the live and the lack of viability of the HK-BCG were controlled by plating serial dilutions

of the BCG stock before and after heat treatment onto plates containing medium. Colonies were counted after a 21 days incubation period at 37 ° C, in an atmosphere containing 9% CO2. No colony forming units (CFUs) could be detected in the stock solution after heat treatment.

3.7 Treatment of mice with helminthes

To address the question whether an Th2 response initiated by helminth infection suppresses or increases OVA-induced Th2 responses C57BL/6 mice were infected intrapreitoneally with 1,000 L3 larvae of the helminth *Nippostrongylus brasiliensis*. Helminths initiate a strong Th2 response characterized by eosinophilia and secretion of IL4, IL-5 and IL-10 by T cells, first in the lung and then in the gut [60].

3.8 Infection of mice with influenza A virus (II)

Bacterial infections induce profound Th1 responses. The administration of certain bacteria or their products may therefore be used as a vaccine or as components of vaccines aimed inhibiting Th2 responses. On the other hand several infectious agents, such as respiratory pathogen viruses are described to trigger asthmatic exacerbations in atopic individuals with pre-existing allergen exposure.

To address the question whether viral infection increases an ongoing allergic response we infected BALb/C and C57/BL6 mice with 30 µl PBS (phosphate-buffered saline) containing 2x105 EID50 (egg infectious dose 50%) of influenza A (Flu) virus intranasally in different time points. To imitate ongoing allergic sensitization by recurrent allergen exposure, mice were challenged with intranasal OVA once weekly as described earlier.

3.9 Determination of active and passive cutaneous anaphylaxis (II)

We hypothesized that virus-specific IgE antibodies may bound to the Fcε receptors of Langerhans cells in the skin evoking the degranulation of the cell, if the mice has already been infected with the virus and rechallenged intradermally. Active cutaneous anaphylaxis was tested 3 weeks after Flu infection. BALB/C and C57/BL6 mice were injected intravenously via the tail vein by 0.5% Evans Blue dye in PBS. Subsequently the skin of the belly was shaved and 50 µl PBS containing 500 µl/ml Flu antigen or PBS alone was injected

intradermally. After 15 min the mice were killed and the skin was stripped off. Positive reactions towards virus antigen resulted in mast cell degranulation and fluid extravasation, which led to the formulation of a blue patch around the injection site. The size of the patch was scored from 0 to 2 by two independent observers.

For detection of passive cutaneous anaphylaxis serum of noninfected control mice or flu-infected mice was collected 3 weeks after infection and pooled. A part of flu-infected serum was depleted from IgE antibodies. Subsequently, groups of noninfected mice were injected intradermally with 50 μl of the various serum pools at 1:5 dilution into premarked sites on the shaved belly. Cutaneous anaphylaxis was measured 2, 24 and 48 h after injection.

4 Results

4.1 Experiments exploring the effects of bacterial infection on allergen-induced airway eosinophilia

4.1.1 Kinetic study of ovalbumin-induced inflammatory response

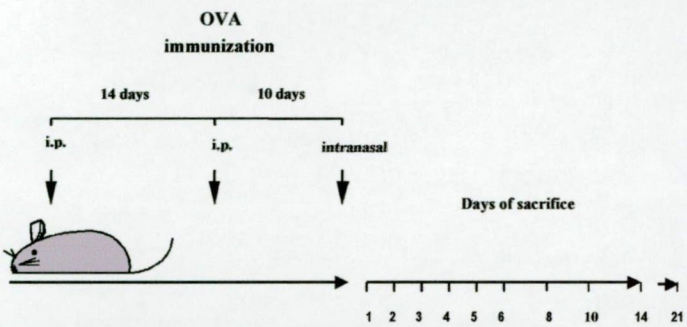

Figure 9. *Ovalbumin immunization kinetic study protocol*

For determination of the ideal time point when the inflammatory cells reach the highest number in the airways and the peak concentration of pro-inflammatory Th2 cytokines is present in the lung, a series of kinetic studies had to be performed. For the model of allergic immune response, the above-mentioned OVA immunization protocol was used in C57Bl/6 mice. Five mice were harvested on each day after the OVA intranasal instillation as indicated in **Figure 9.**

At analysis, mice were sacrificed, the trachea canulated, and BAL performed. BAL cells were counted and spun onto glass slides using a citospin and the cells were stained and counted by microscope. Blood serum samples were drawn and OVA-specific IgE and IgG1 immunoglobulins were determined by ELISA. Single cell suspensions from the MLN were prepared and cultured. Supernatants were harvested and tested for the presence of cytokines, such as IL-4, IL-5 and IFN-γ by ELISA.

Following OVA intranasal challenge, BAL eosinophils were significantly elevated from days 3 to 8 and decreased from day 10 (**Figure 10**). OVA immunization resulted in significantly

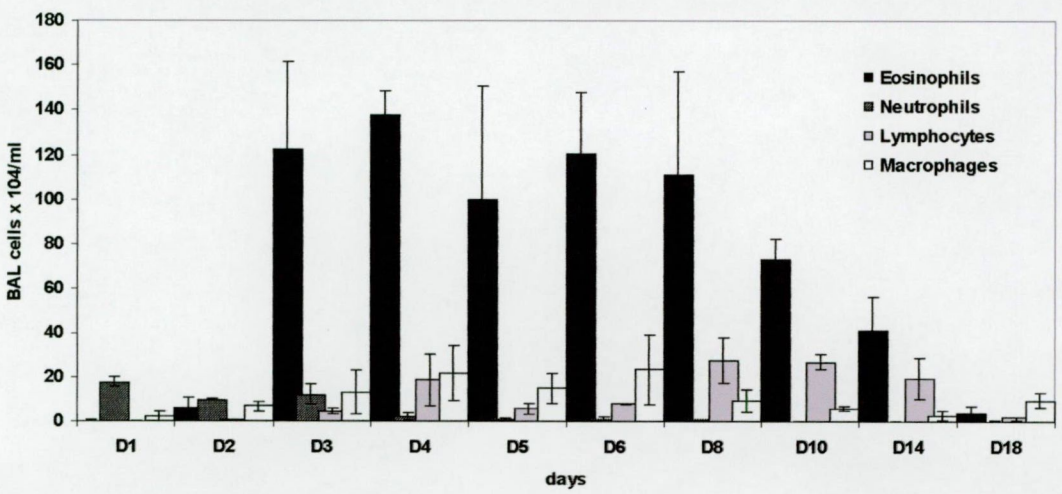

Figure 10. *BAL F Inflammatory cells in OVA-induced airway inflammation. Number of eosinophils shows a rapid increase from*

higher IL-5 levels already on day 2 after OVA intranasal boosting and remained until day 14 (data not shown). Moreover eotaxin, which is the chemokine responsible for airway eosinophil recruitment, reached its peak concentration on day 2, though the levels showed a rapid decrease on day 3 (**Figure 11 A**). Serum OVA-specific IgE showed a significant increase from day 4, reaching the highest level on day 10 (**Figure 11 B**).

The kinetic study showed that the best time point for dissection for this model is day 6 after OVA intranasal challenge. Based on this model a series of studies was designed to investigate the influence of different infections on allergen-induced immune responses.

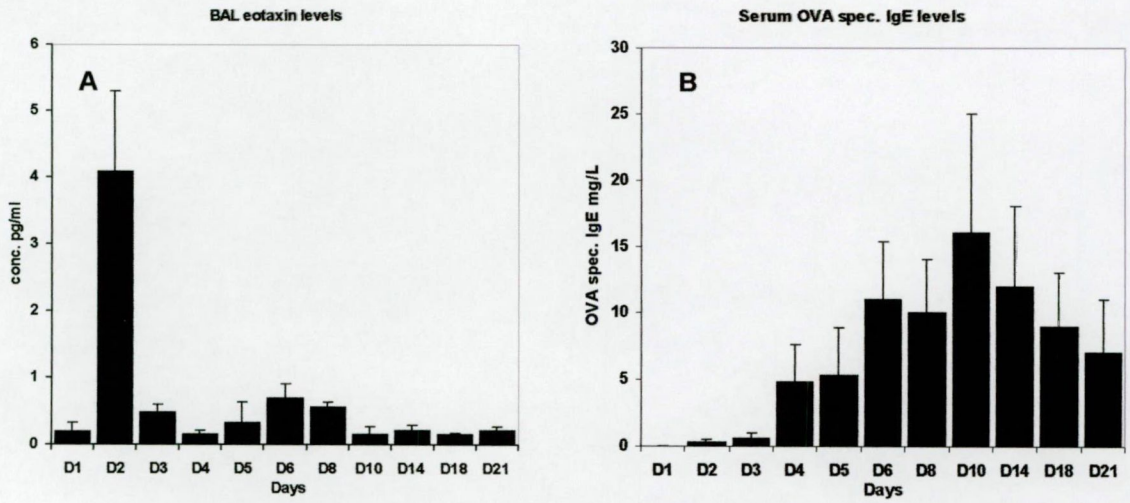

***Figure 11. A**: BALF eotaxin levels at different days in OVA-kinetic study. **B:** Serum OVA-specific IgE levels at different days in OVA-kinetic study*

4.1.2 Application of HK-BCG into the lung inhibits the development of OVA-induced airway eosinophilia. (I, III)

As discussed above, the suppressive effect of BCG on OVA-induced airway eosinophilia is already known. The purpose of the further studies was to investigate whether dead bacteria, or bacterial fragments, are able to evoke the same effect. Therefore C57Bl/6 mice were pre-treated intranasally with $2x10^6$ CFUs of BCG, HK-BCG or PPD four and eight weeks prior to OVA intranasal challenge. OVA-immunization protocol was used as control (described earlier). Mycobacteria were killed by incubating twice for one hour each time at 80 °C in a warm water bath. To investigate whether the inhibition of allergic immune response was present in the absence of bacterial cellular wall antigens (possibly CpG motifs), PPD,

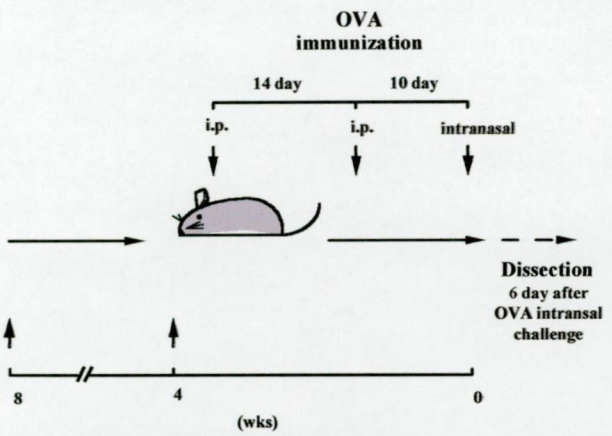

Figure 12. *Experimental protocol for BCG, HK-BCG, PPD-OVA immunization experiments*

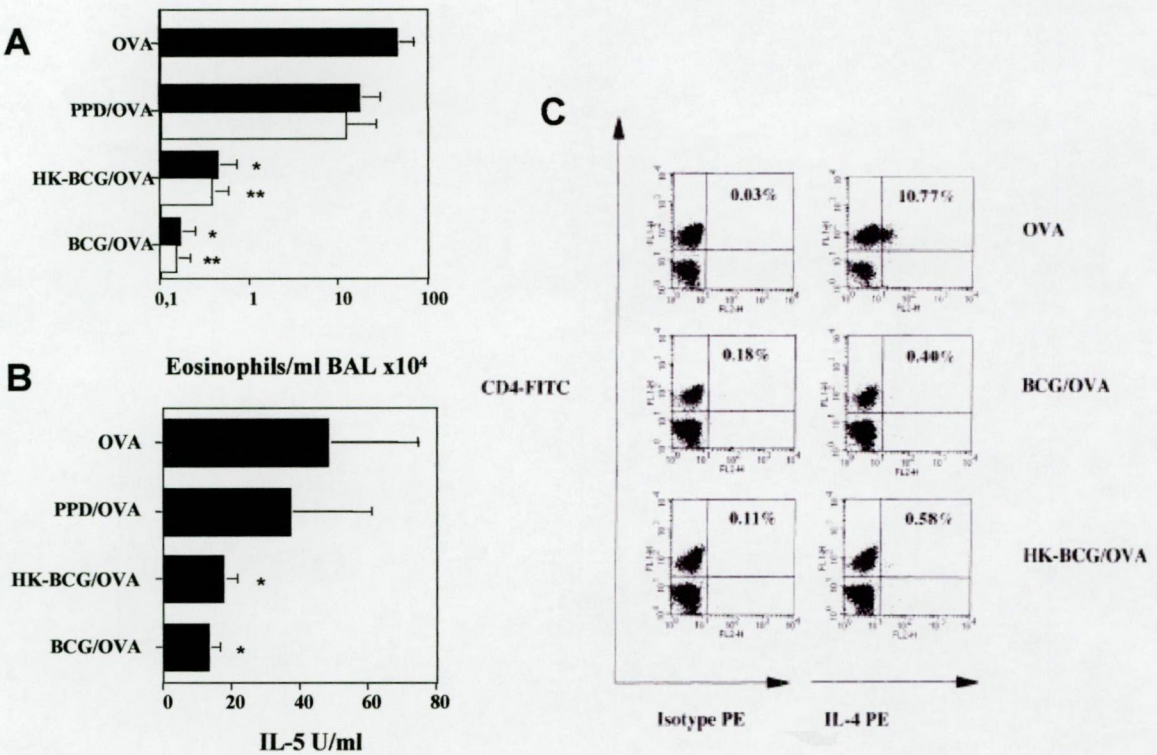

Figure 13. A: *Shown are the average numbers of eosinophils +/.SD present in the BALs of 5–6 mice/group (A).P < 0.05 .P <0.001, compared to values obtained in OVA-only treated mice.* ***B:*** *Shown is the average amount of IL-5 present in the MLN cultures from six mice per group with SD determined by ELISA. P < 0.05, compared to values obtained in mice only immunized with OVA.* ***C:*** *Shown are FACS-stainings gated on CD4+ T cells representative of six mice per group. The percentage of CD4+ T cells positively stained for IL-4 production is indicated*

which contains smaller fragments of cellular wall elements, was also applied to a group of mice.

To summarize the results, it can be clearly stated that an intranasal vaccination with BCG or

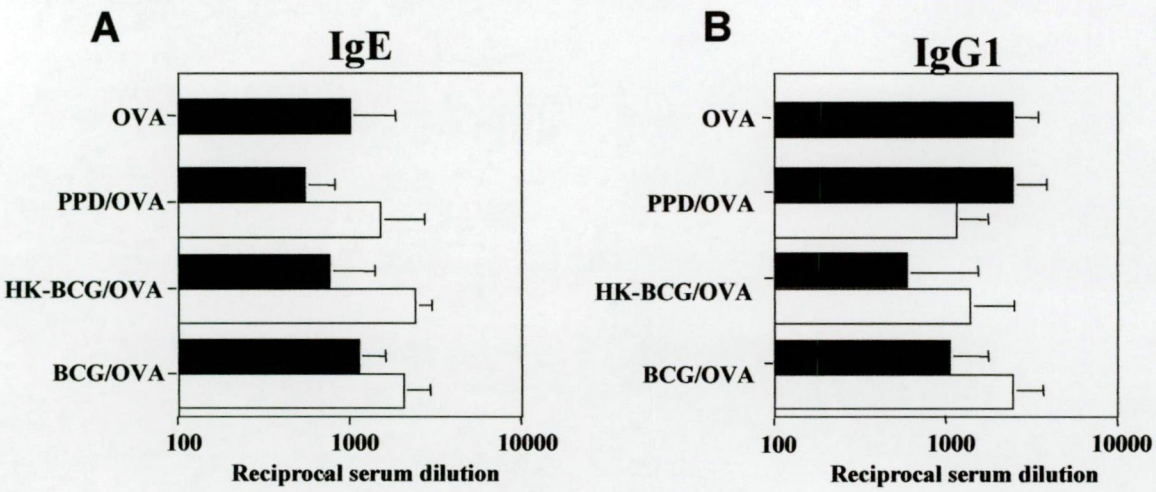

Figure 14. *Treatment of mice with BCG and HK-BCG did not inhibit the development of OVA-specific IgE and IgG1 antibodies. Serum was prepared 6 days after OVA airway challenge and OVA-specific IgG1 and IgE antibody levels were determined. Values represent mean serum IgE (**A**) and IgG1(**B**) titers of 5–6 mice/group (4 weeks BCG/PPD-treated mice: closed symbols; 8 weeks BC/PPD-treated mice: open symbols) with SD.*

HK-BCG, but not PPD, given four or eight weeks prior to allergen airway challenge, resulted in a strong suppression of airway eosinophilia. This effect correlated with reduced levels of IL-5 producing Th2 cell numbers present in the airways of OVA-challenged mice (**Figure 13 A-C**).

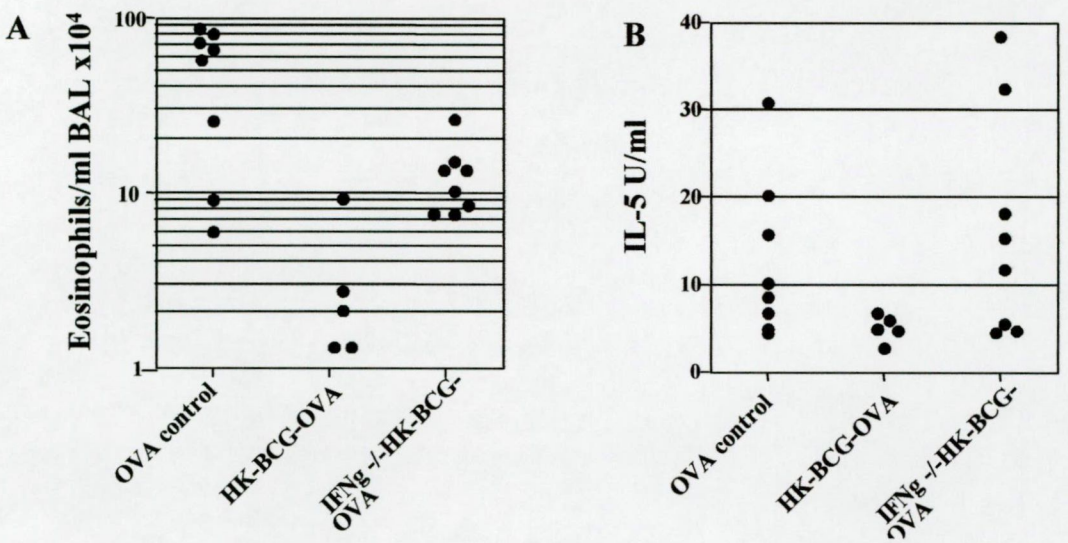

Figure 15. *HK-BCG-induced suppression of airway eosinophilia and reduction of IL-5 secretion by MLN cells was dependent upon the presence of IFN-γ.*
*Shown are the numbers of eosinophils present in the BALs (**A**) and the amount of IL-5 secreted by the MLN cells after in vitro restimulation with anti-CD3/IL-2 of individual mice (**B**).*

BCG and HK-BCG induced suppression of airway eosinophilia and reduction of Th2 cell numbers seems to be a localized to the lung, since the systemic Th2-dependent production of OVA-specific IgG1 and IgE by the B cells was not reduced by the intranasal application of the live and heat-killed mycobacterium (**Figure 14 A, B**).

To address the question whether IFN-γ was also involved in HK-BCG-induced inhibition of airway eosinophilia, IFN-γ deficient and control mice were subjected to the same OVA-immunizations protocol and infected four weeks prior to the intranasal challenge, resulting in a strongly reduced suppression of airway eosinophilia in IFN-γ deficient mice (**Figure 15 A, B**).

It is somewhat surprising that there was only a slight difference in the suppressive effect of BCG and HK-BCG because the histological analysis showed more extensive inflammation in the BCG-treated mice. One possible explanation is that the $2x10^6$ CFU BCG and HK-BCG was much more than required for effective suppression. Therefore OVA-immunized mice were treated with different doses of BCG and equivalent amounts of HK-BCG. The BCG was very efficient in inhibiting airway eosinophilia at a dose of 2×10^6, 2×10^5 and 2×10^4 CFU, whereas the HK-BCG showed a maximal suppressive effect only when the highest dose (equivalent to 2×10^6 BCG) was used. This result clearly demonstrates that the application of live BCG in comparison to HK-BCG is more efficient in suppressing the development of allergen-induced airway eosinophilia in lower doses (**Figure 16**). This finding could be explained by the fact that live bacteria are able to multiply, causing more extensive inflammatory reaction. Another possible explanation is that heat killing damages the surface LPS molecules, hence diminishing the antigenity effect.

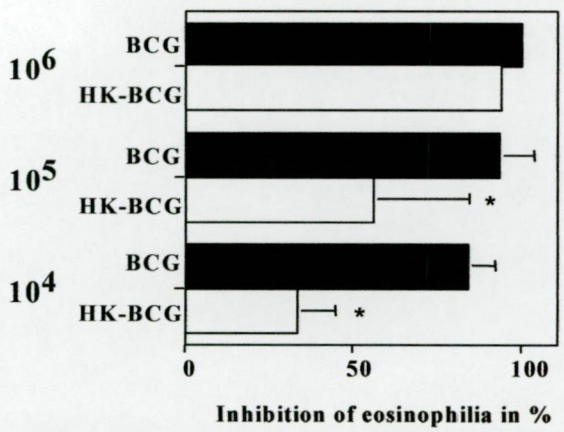

Figure 16. *Shown is the mean suppression of airway eosinophilia (%), in relation to eosinophil numbers detected in OVA control mice (using the mean of six mice), with SD of six mice per group. P < 0.05, compared to the percentage of inhibition obtained using 2×106 live BCG.*

Histological sections made from the lungs of OVA-only versus HK-BCG–OVA-treated mice clearly show a significant decrease in mucus production compared to the HK-BCG-treated group (**Figure 17 A, B**).

Taken together, the results of these experiments clearly demonstrate that applications of heat-killed mycobacteria are able to suppress ovalbumin-induced airway inflammation in mice. This effect is localized to the target organ, in this case the lung, and no longer detectable in any other organs. HK-BCG is less effective in suppressing airway eosinophilia in lower concentrations compared with equivalent amounts of BCG. The suppression of airway eosinophilia appears to be Th1 immune response-driven, as no suppression of OVA-induced eosinophilia was detectable in IFN-γ-deficient mice.

The major objective of these investigations was to identify a possible vaccination method against the development of allergic disorders such as bronchial asthma. However, this approach may harbor potentially negative side effects that need to be ruled out before vaccinating young children, who are the best candidates for the primary prevention of allergic disorders.

Using live bacteria as a vaccine is potentially hazardous because the host organism could become infected. On the contrary, dead bacteria or fragments of the bacterial wall containing CpG motifs have the potential to trigger immune-responses similar to those triggered by infections; therefore, they could be suitable candidates for vaccine development in the future.

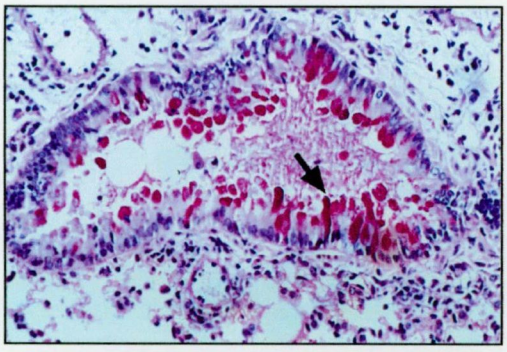

Figure 17 A: *Mucus produced by goblet cells in OVA-induced allergic reaction. PAS staining*

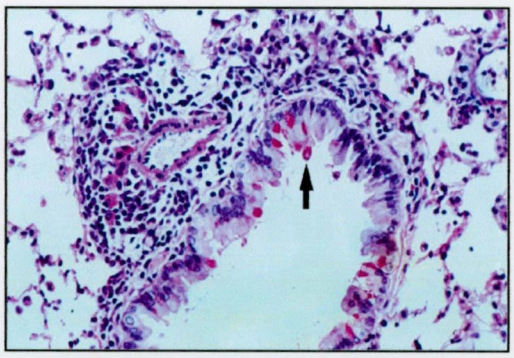

Figure 17 B: *Mucus produced by goblet cells in HK-BCG pretreated, OVA-induced allergic reaction. PAS staining*

According to our observations, as HK-BCG was effective in suppressing allergic reaction up to eight weeks after application, bacterial surface antigens are able to induce long-term prevention against allergic reactions. However, the extent of this suppression is undefined. Moreover, the effects of multiple allergen stimuli on the protective capacity of bacterial antigens are also unclear.

Another problem is that this method as a potential vaccination could only be beneficial prior to the maturation of the immune system in an atopic individual. When an atopic phenotype is already present, these bacterial antigen insults could prove potentially dangerous by triggering the development of allergic symptoms.

4.2 Experiments exploring the effects of helminth infection on allergen-induced airway eosinophilia

4.2.1 Kinetic study of helminth infection-induced inflammatory response

A second series of kinetic studies was designed to investigate the characteristics of helminth infection-induced inflammatory response. The objective was to determine the extent and the timing of the inflammatory response.

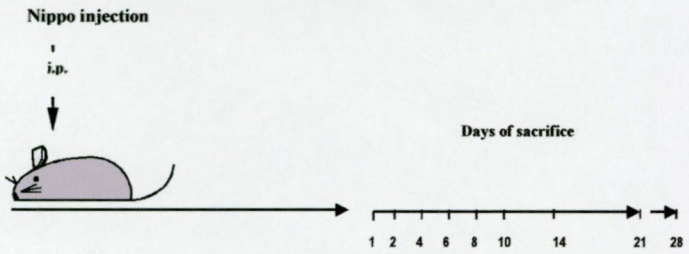

Figure 18. *Experiment design of the helminth infection kinetic study. Mice were infected with Nippo intraperitoneally and harvested 1-28 days after injection.*

For this model, *Nippostrongylus brasiliensis* (Nippo), a metazoic worm, which is a common pathogen among rodents, was used. Approximately 1000 larvae in 1 ml PBS were injected intraperitoneally into C57Bl/6 mice. The animals were sacrificed and dissected on days 1, 2, 3, 4, 5, 6, 8, 10, 14, 21 and 28 after infection (**Figure 18**).

As in the OVA kinetic study, blood, BAL and mediastinal lymphnodes were taken. BAL cells

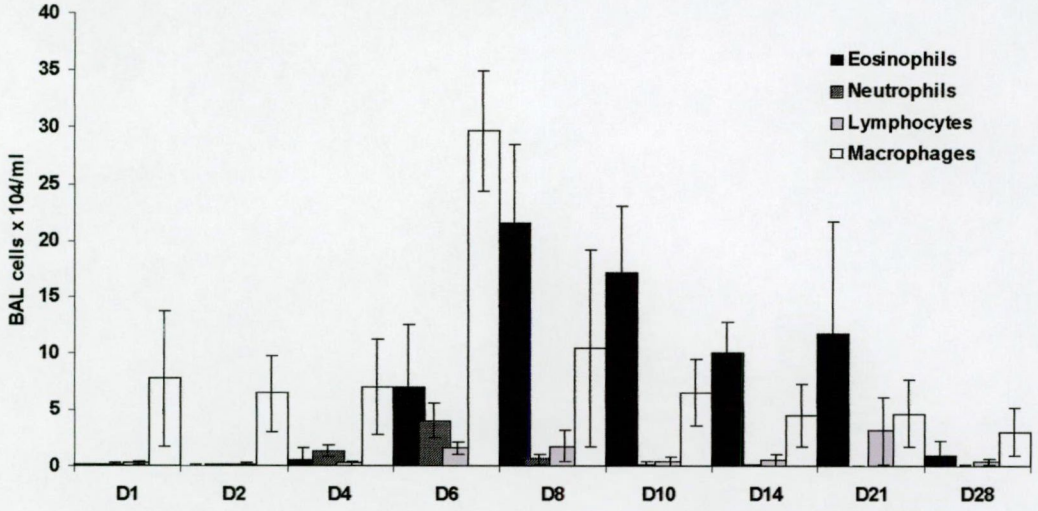

Figure 19. *Nippo kinetic study. Inflammatory cells in BALF after 1-28 days of Nippo infection. Eosinophils are in the highest numbers on day 8-10.*

were counted and the same cytokines and immunoglobulins were determined as before. The following Figures show the levels of peripheral blood immunoglobulin, BAL cytokine levels and numbers of eosinophils in BALF (**Figure 19; 20. A-D**). The Nippo-induced inflammatory

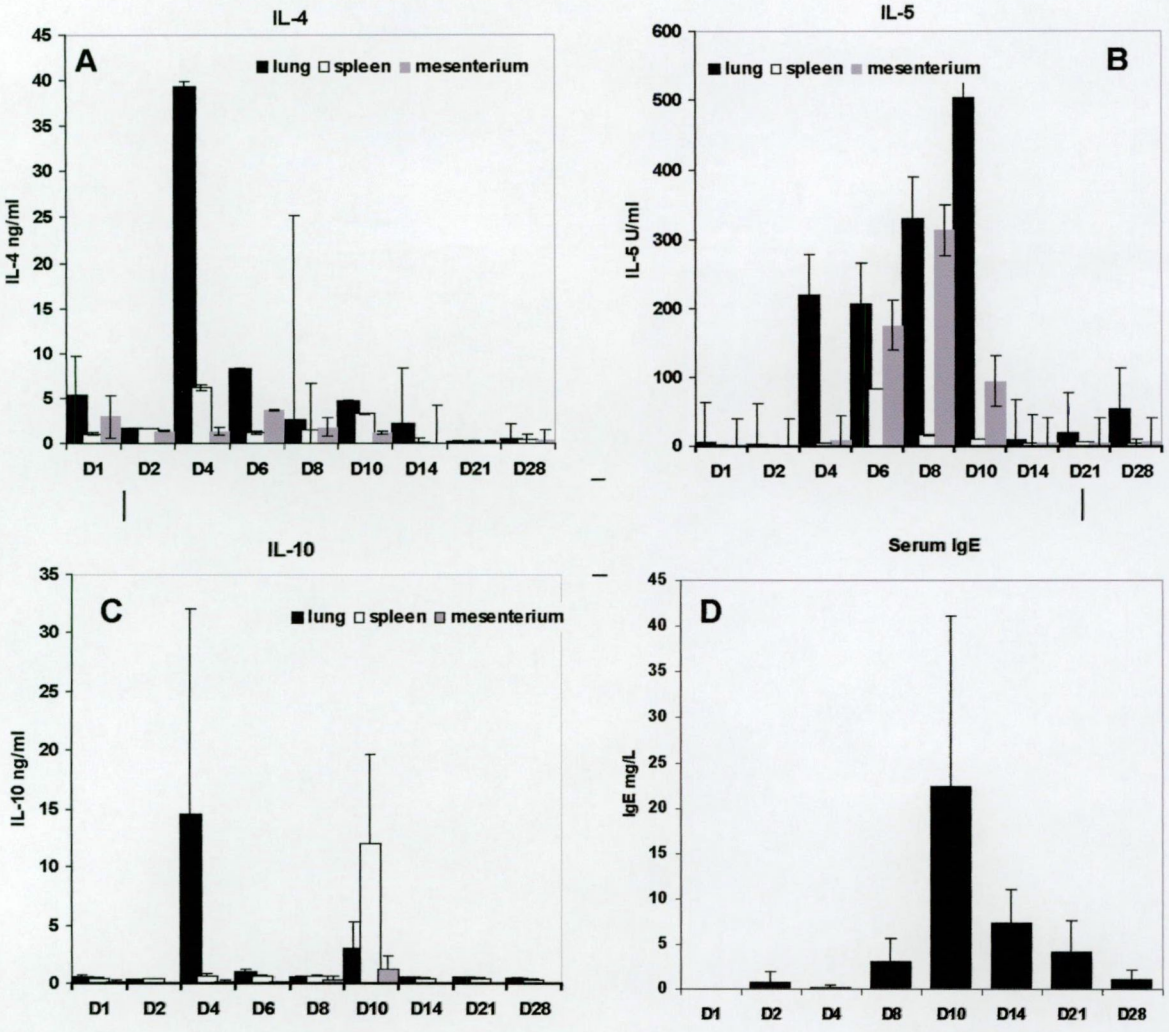

***Figure 20**. Cytokines (**A**: IL-4; **B**: IL-5; **C**: IL-10) were determined in the supernatants of T cell cultures from MLN. **D**: Nippo-specific IgE levels were measured in peripheral blood sera.*

response reached the highest intensity between days 8 and 10 after injecting the larvae. The Th2-type inflammatory reaction had disappeared by day 21. In consequence of this study, the timing of infections in different groups was adjusted to the results in the further experiments, where helminth infection was applied prior to OVA immunization.

4.2.2 The effects of Nippostrongylus brasiliensis infection on ovalbumin-induced airway eosinophilia

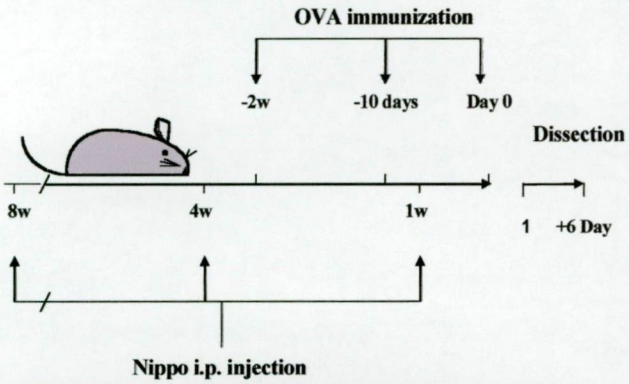

Figure 21. *Experiment design for Nippo infection-OVA immunization experiment. Mice were infected with Nippo 8, 4 and 1 wk before OVA intranasal challenge. The mice received OVA immunization treatment according to the protocol mentioned above. Mice were harvested 6 days after OVA intranasal challenge.*

After completion of the Nippo-kinetic experiments a series of combined studies was designed to explore the effects of a pre-existing helminth infection on allergic immune response. The objective of these studies was to determine whether a pre-existing helminth infection would increase or decrease an unrelated allergen-induced eosinophilia in the airways. C57-Bl/6 mice were infected with Nippo 8, 4 and 1 week(s) prior to OVA immunization and intranasal challenge. BALF were collected 6 days post-challenge and cellular and humoral immune responses were measured (**Figure 21**).

For Nippo infection 1000 larvae in 1ml PBS were injected into the mice intraperitoneally. For OVA immunization, the same protocol was used as described before.

Our results revealed decreased IL-5 production in the cell cultures made from mediastinal lymphnodes taken from mice infected with Nippo 8 and 4 weeks (but not 1 week), before OVA sensitization (**Figure 22 A**). Increased IL-5 protein levels and decreased IFN-γ protein levels were also observed in the BALF (data not shown). There was, however, no increase, but rather a significant decrease in airway eosinophil accumulation in mice infected with parasites 4 and 8 weeks prior to sensitization with OVA as compared to the group infected 1 week prior and to mice exposed to OVA alone (**Figure 22 B**).

According to our observations in the kinetic study, the Nippo-induced immune response has already ceased by the time OVA intranasal challenge occurs, yet there is still suppression of the development of allergic reaction. On the contrary, a significant increase can be observed in the numbers of BALF eosinophils in the group infected 1 week prior to OVA challenge, suggesting that Nippo-induced Th2 immune response may contribute to the increased allergic Th2 immune response.

Furthermore, eotaxin levels in the lungs induced by OVA were suppressed in mice infected with the parasite 4 and 8 weeks before OVA sensitization, but not in the group infected 1

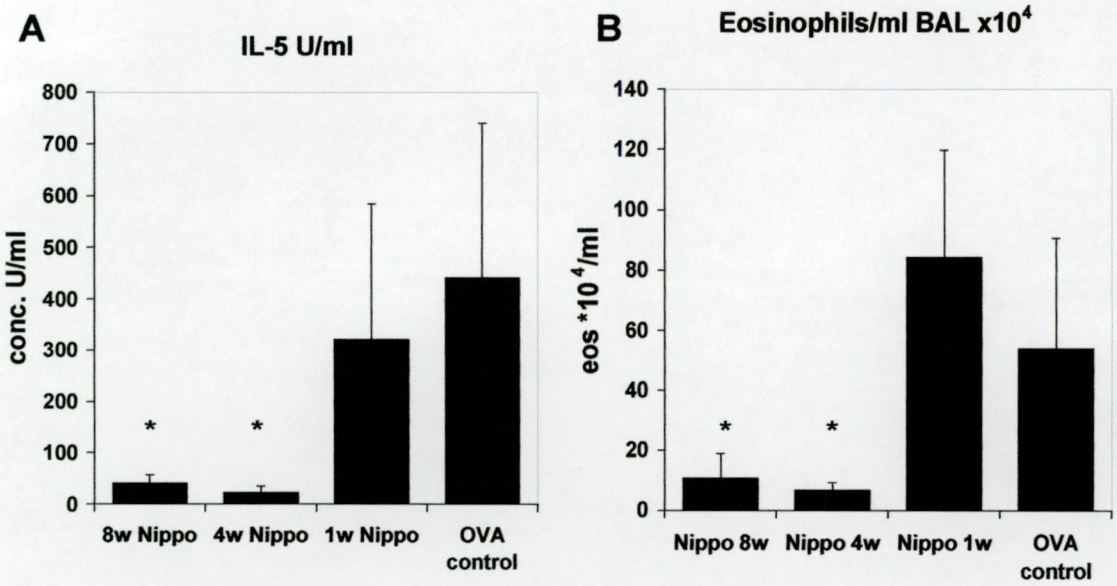

Figure 22. A: *Increased IL-5 levels in the Nippo 4 and 1 week prior to OVA intranasal immunization infected groups versus OVA controls.*
B*: BAL eosinophilia is suppressed in the groups infected 8 and 4 weeks before OVA intranasal challenge. Interestingly a significant increase can be observed in the group, which was Nippo infected one week before OVA immunization. * P≥0.005*

week prior (data not shown).

These results suggest that a pre-existing helminth infection may potentiate a systemic Th2-type response, yet simultaneously suppress in the lungs allergen-specific IgE responses and eotaxin production in response to subsequent exposure to allergens depending on the time of infection.

Our results revealed confounding counteraction between two independent Th2 immune responses that are similar in nature but different in origin.

4.3 Major Histocompatibility Complex class I molecules are necessary for the development of OVA induced airway eosinophilia

We addressed the question of whether MHC I molecules are necessary for successful allergen presentation in antigen-specific immune responses. To find an answer to this question, an experiment was designed where the strength of OVA-induced airway eosinophilia was compared in MHC class I-deficient mice and normal controls. MCH I knockouts and C57/Bl6 mice as controls were immunized with OVA according to the same protocol used in the former experiments. Six days after OVA intranasal challenge the mice were dissected and BALs were performed. Our results show that MHC I-deficient mice failed to develop eosinophilia after OVA immunization compared to the controls [61] (**Figure 23 A**). Interleukin-

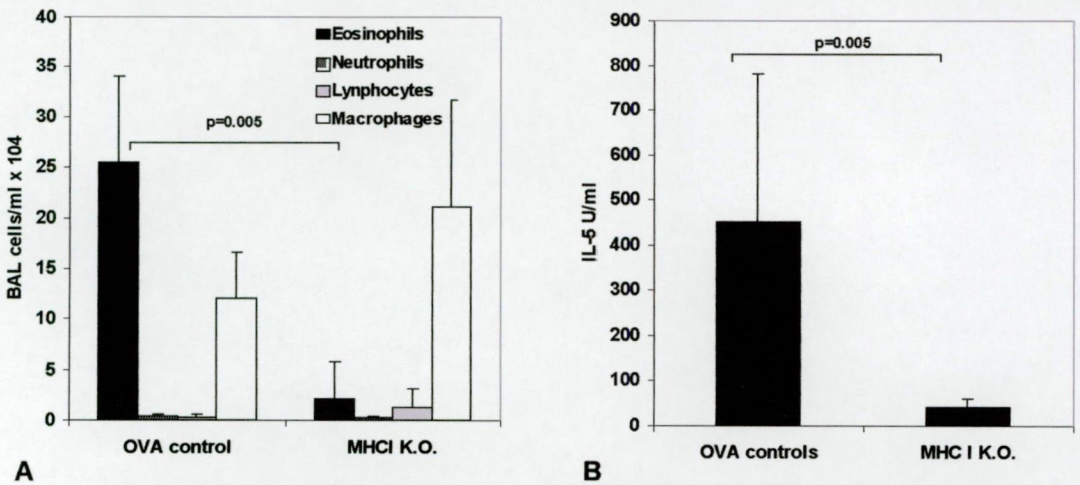

Figure 23. A: *BALF inflammatory cells in OVA controls and MHC I knockouts. Eosinophis were increased in OVA controls, whereas no change was detected in the numbers of eosinophils in the MHC class I knockout mice.* ***B:*** *IL-5 levels in the supernatants of the T cell cultures from the MNL. Significantly higher levels of IL-5 were measured in OVA controls as compared to MHC class I knockouts.*

5 levels measured in the supernatants of the cell cultures made from the draining lymphnodes of the lung were significantly higher in OVA controls compared to MHC I-deficient mice (**Figure 23 B**).

4.4 Influenza infection leads to Flu antigen induced cutaneous anaphylaxis in mice (II)

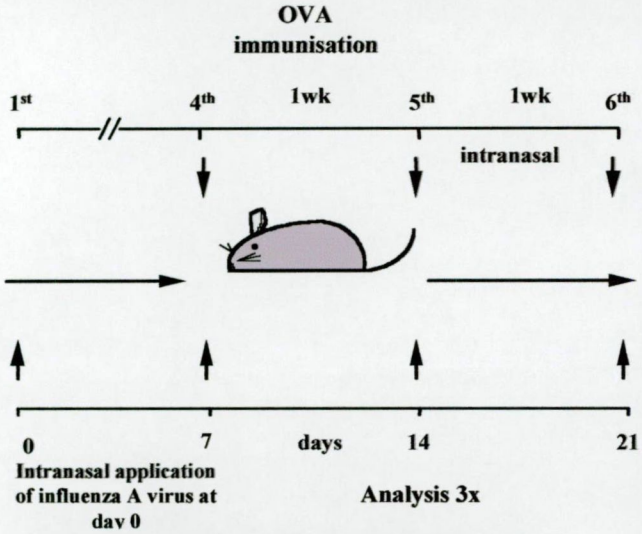

Figure 24. *Experimental design for intranasal OVA sensitization and flu infection experiments.*

Viral infections generally induce strong Th1 immune responses that may inhibit the development of allergen-specific Th2 cells via the production of IFN-γ [62]. Therefore it has been proposed that viral infections should inhibit the development of atopic disorders. Clinical observations and experimental data, however, suggest that the opposite happens [63-67]. A possible mode of action is that viral infections

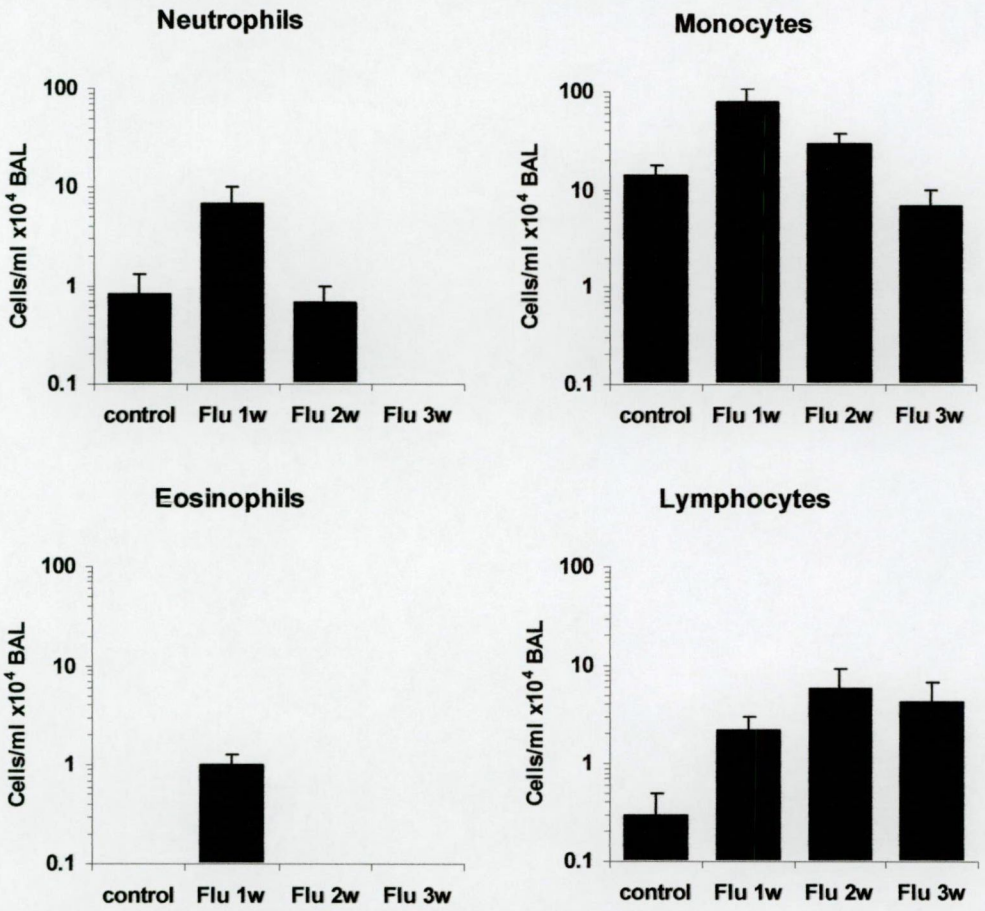

Figure 25. *Infection of mice with flu virus induces a strong influx of lymphocytes, macrophages, and neutrophils but recruits only few eosinophils into the lung. Mean cell1 counts x IO⁴/ml BAL fluid of the different cell types from six to seven individual mice per group with SD is shown (*statistical significance in comparison with uninfected controls).*

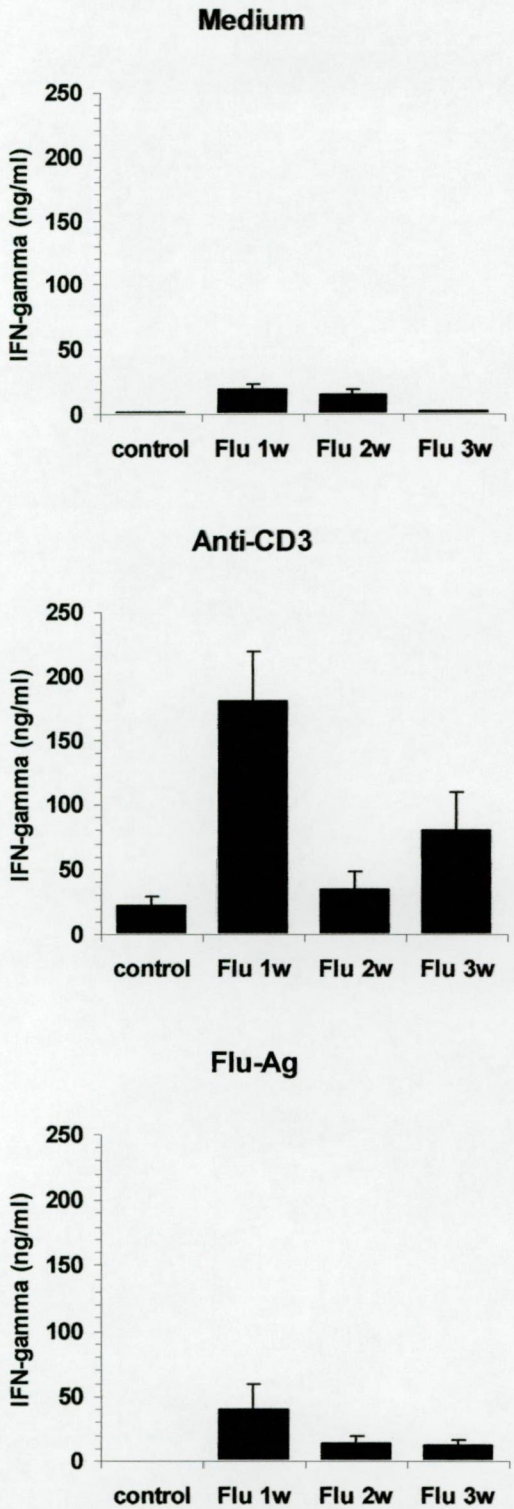

Figure 26 *MLN cells obtained from the lungs of mice previously infected with flu virus show a strong production of IFN-γ, after in vitro restimulation with Flu-Ag or anti-CD3 in combination with IL-2. Shown are the mean amounts of IFN-γ from five to six mice per group with SD (*statistical significance in comparison with uninfected controls).*

induce a minor Th2 response in parallel to the strong Th1 response, leading to the production of virus-specific IgE antibodies. These antibodies could directly induce mast-cell degranulation when encountering viral antigens, thereby causing exacerbation of allergic symptoms [67].

To address the question of whether viral infection could lead to mast cell degranulation; mice were infected with influenza A virus and the resulting inflammation in the lung was analyzed histologically. For this purpose, lung sections were prepared before and on days 7, 14, and 21 after infection (see 2nd attachment, Materials and Methods, **Figure 24**) [59]. Our results revealed a strong Th1 type inflammation with perivascular infiltrates dominated by macrophages, lymphocytes and neutrophils (**Figure 25**).

Single cell suspensions of the MNL were prepared at the same timepoints after infection and left in medium or restimulated with Flu-Ag or anti-CD3/IL-2 *in vitro* to study the cytokine profile of the T lymphocytes in the lung. **Figure 26** shows that T cells produce increased IFN-γ both after Ag specific and polyclonal anti-CD3/IL-2 restimulation.

Low levels of IL-5 could be detected in the supernatants of MLN from 2 wk infected mice 72 h after restimulation with Flu-Ag (data not shown) suggesting that besides a strong Th1

dominated immune response, a small number of Th2 cells were generated during the flu infection in the lung. Serum Th1- associated flu-specific IgGa and IgGb titers showed a strong increase. Surprisingly, Th2 associated IgG1 and some virus-specific IgE antibodies were also detected (**Figure 27**). This raised the question of whether the virus-specific antigens had elicited the allergic symptoms. Therefore we investigated whether the mice infected with flu virus for 3 weeks developed active cutaneous anaphylaxis after re-challenge with Flu-Ag. For this purpose mice were injected i.v. with Evans Blue dye and then with Flu-Ag and PBS intradermally into two separate premarked sites in the skin. After 15 min. the mast cell degranulation induced extravasation of stained blood serum, which led to the formation of a blue patch around the injection site of viral antigens only in the flu-infected mice (**Figure 28**). The lack of this blue patch formation in

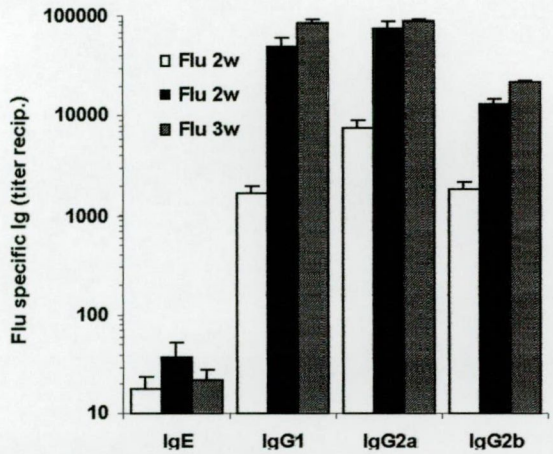

Figure 27. *Mice show a strong increase in flu-specific IgG2a, IgG2b, and IgG1 in the serum after flu infection, but also low levels of virus-specific IgE. Shown are the mean reciprocal values with SD of the serum dilutions, which were 2-fold over the background OD of noninfected mice (Six mice per group).*

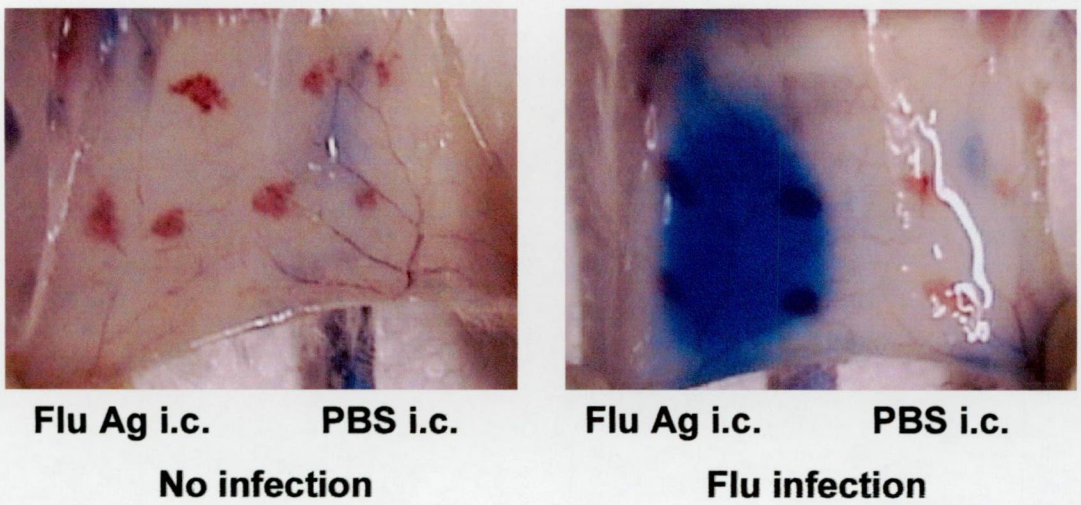

Figure 28. *Mice infected with flu virus show Flu-Ag-specific active cutaneous anaphylaxis. Active cutaneous anaphylaxis was tested in uninfected mice or in mice that had been infected for 3 weeks with flu virus. Mice were injected i.v. with Evans Blue Dye. Subsequently the skin of the belly was shaved and Flu-Ag or PBS was injected intradermally into the skin. After 15 min the intensity of bluing as an indicator for the intensity of mast cell degranulation was scored as follows: 0=no bluing, 1=slight bluing, 2=strong bluing.*

non-infected mice indicates the lack of mast cell degranulation after viral antigen challenge. After intradermal Flu-Ag injection, mice that had been infected with flu virus showed a much stronger mast cell degranulation than noninfected controls (**Figure 29 A**).

We wanted to exclude nonspecific, virus-altered mast cell reactivity and to prove that specific IgE molecules are responsible for the mast cell degranulation effect. Therefore, we passively transferred serum from flu-infected mice into non-infected mice and analyzed for passive cutaneous anaphylaxis according to the protocol mentioned above. For this purpose, noninfected mice were injected intradermally either with 50 µl normal, or IgE depleted serum from mice, which had been infected with flu virus for 3 weeks. Local anaphylaxis was measured at the sites of the previous injections at 2 h, 24 h, and 48. Intradermal Flu-Ag challenge resulted in similar mast cell degranulation in mice which were injected with the serum of flu-infected mice. In contrast, the passive cutaneous anaphylaxis was significantly lower in mice that had been treated with IgE-depleted serum from flu-infected mice or with noninfected sera (**Figure 29 B**).

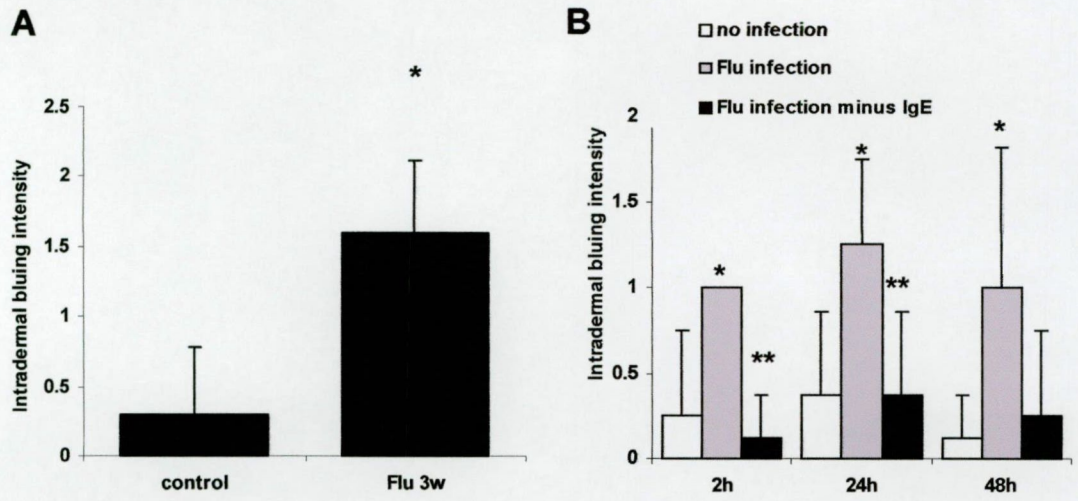

Figure 29 ***A:*** *Average bluing intensity with SD of the different groups of mice after active cutaneous anaphylaxis (10 mice per group; *statistical significance in comparison with uninfected controls).*
B: *Passive cutaneous anaphylaxis. For detection of passive cutaneous anaphylaxis groups of mice were injected intradermally with pooled sera of noninfected mice, 3 wk infected mice and IgE depleted sera of 3 wk infected mice as indicated. Cutaneous anaphylaxis was measured 2, 24, and 48 h later in the sites of previous injection as described above. Data shown are the average bluing intensity with SD (four mice per group). (* statistical significance in comparison with uninfected controls; ** statistical significance in comparison with flu-infected mice).*

Taken together, the results of this study showed that viral infection can mediate mast cell degranulation by the production of virus-specific IgE antibodies, which leads to the exacerbation of allergic symptoms.

5 Discussion

5.1 Influence of infectious diseases on atopy

It is generally accepted among experts, that there is a difference in the increase of atopic disorders in developed countries versus developing countries [24,68]. The hypothesis that the genetic background in different populations could be distinct, however, does not provide a satisfactory explanation since there is an increase in the incidence of atopic diseases in people from developing countries exposed to Western living conditions [69]. The change in lifestyle towards westernization might also be a reason for the observed increase in allergic diseases in the former East German population [70]. The steady decline of chronic infectious diseases in the developed world could be accountable for the increase of atopy. Recent epidemiological studies provide support for this hypothesis by demonstrating an inverse relationship between atopy and immunization/infection with mycobacteria, measles, and hepatitis A [71,72].

5.1.1 Can infectious diseases suppress the development of atopic disorders?

Infectious diseases inducing Th1–type responses lead to the production of interferon γ (IFN-γ) [73,74]. IFN-γ plays a major role in the suppression of Th2 immune responses[62,75-78]. A number of reports support the hypothesis that infections inducing Th1 environment might impede the development of allergen-specific Th2 cells. The development of allergen-induced airway eosinophilia has been reported to be suppressed by infection with *M. bovis -BCG* in mice [79].

One possible mode of action is that naive T cells after activation, in the absence of IL-4 (which augments Th2 response), express both α- and β- chains of IL-12 receptors, thus facilitating IL-12-mediated Th1 cell development. Expression of the β subunit is required for recruitment and activation of the STAT proteins involved in IL-12 signaling [80]. The expression of the β- chain, however, is repressed in the presence of IL-4, resulting Th2 cell development. On the contrary, INF-γ overrides the IL-4-induced inhibition of the β-chain expression, resulting Th1 development [81].

Expression of transcription factors necessary for Th2 cell differentiation can be suppressed by infection and production of transcription factors needed for Th1 generation can be induced by infection. Moreover, Th1 immune responses could also inhibit the expression of chemokines induced by allergic inflammation, which are essential for the successful recruitment and

homing of eosinophils and Th2 cell into the site of inflammation (**Figure 30**). One of these chemokines is eotaxin, which is produced predominantly by epithelial cells and alveolar macrophages and expressed during allergic inflammatory responses [82,83]. As the chemokine receptor 3 (CCR3) is the receptor for eotaxin, which is expressed only by Th2 cells, it may well be that Th1 immune responses in the lung could down-regulate the kinetics of eotaxin expression. [84,85].

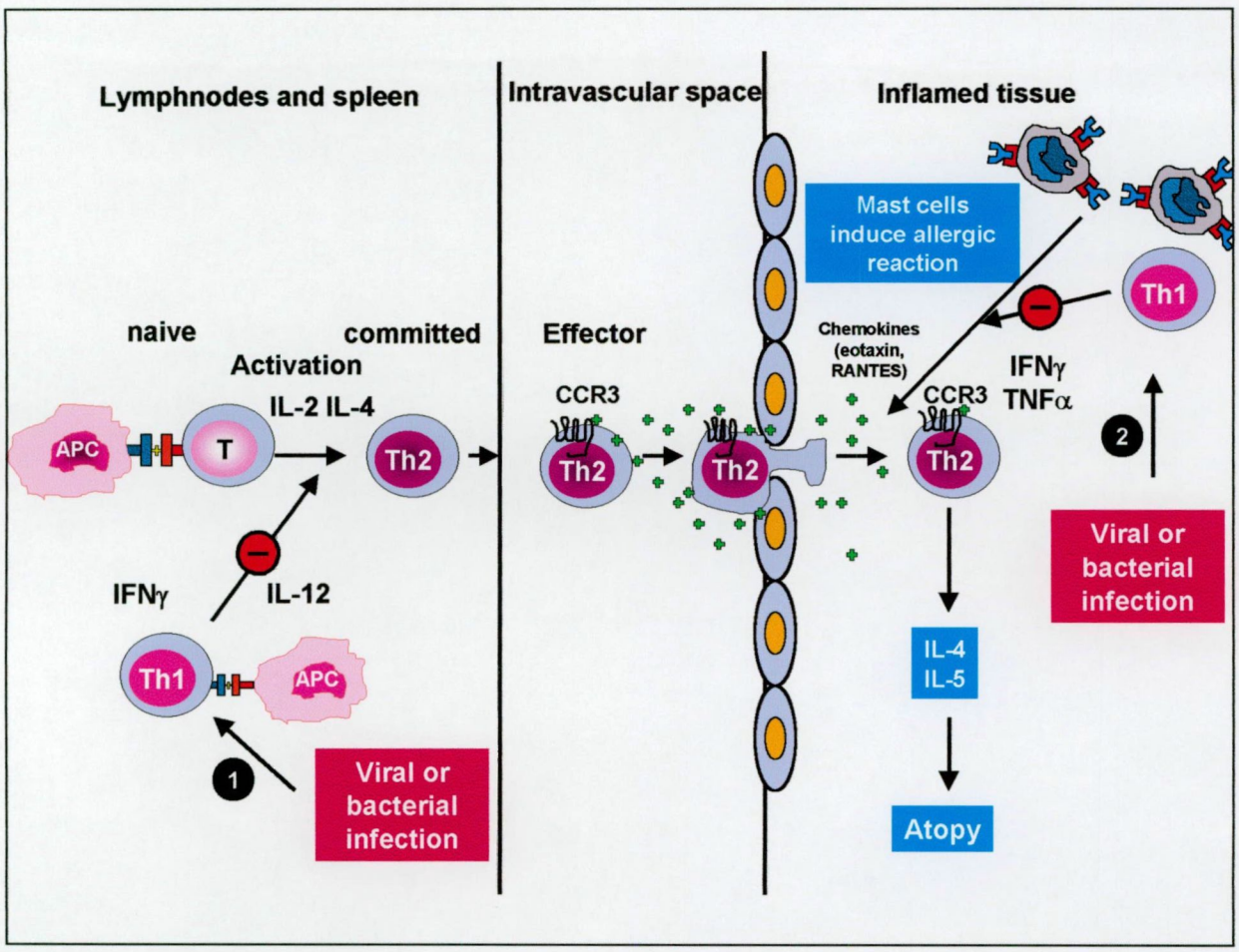

Figure 30. *Proposed inhibition of atopic disorders by infections that induce Th1 immune responses. APCs take up allergens and present peptides to naive T cells in the spleen and lymphnodes. Genetic and environmental factors then determine whether IL-4 is present during the priming and proliferation of the naive T cells, which would lead to the generation of allergen-specific Th2 cells. Effector Th2-type cells express the CC chemokine receptor 3 (CCR3) and circulate in the bloodstream. Allergic inflammation in the tissue increases the production of eotaxin. Eotaxin binds to the CCR3 on the circulating Th2 cell, enabling the cells to home into the inflamed tissues. Upon stimulation with allergen, the Th2 cells secrete large amounts of IL-4 and IL-5. The production of IL-4 induces the production of allergen-specific IgE, which binds with high affinity to mast cells. IL-5 production leads to the development of eosinophils in the bone marrow and is involved in the recruitment into the airways. Infections inducing Th1 immune responses may inhibit the development of allergen-specific Th2-type cells in the spleen and lymph node by providing IFN- γIL-12 at the site of naive T-cell priming (1). Furthermore, Th1 responses at the site of allergic inflammation may inhibit the production of chemokines necessary for the efficient homing of Th2 cells into these sites, further suppressing the development of atopy (2). Taken and modified by permission from: Erb K J. Immunol Today1999;20:317*

The effect of infections to suppress allergic inflammation seems to be localized. Intranasal BCG infection, for example, strongly inhibits Th2 response in the lung, but does not inhibit the systemic allergic response [86,87]. These findings suggest that the infection needs to occur at the same site as the allergic response to have the maximal suppressing effect. The neonatal immune response of non-atopics is biased towards Th2 responses, which then shift towards Th1 responses during the first years of life. By contrast, children who become atopic do not lose the Th2 response.

5.2 Both live and dead bacteria mounted against OVA-induced airway eosinophilia are able to suppress allergic inflammation (I, III)

Our results clearly demonstrate that the application of live and HK-BCG but not PPD into the lung of mice suppressed the development of OVA-induced airway eosinophilia. Furthermore, mucus production after allergen-challenge was also inhibited by the application of live and HK-BCG. The suppression of airway eosinophilia and goblet cell metaplasia correlated with an almost total lack of Th2 cells present in the airways of the live and HK-BCG treated mice. This suggests that the application of live and dead mycobacteria inhibited the recruitment and or expansion of Th2 cells homing into the lung after OVA airway challenge. The observation that T cells from the MLN of live and HK-BCG treated mice showed only a 2 to 3-fold reduction in IL-5 production after in vitro stimulation argues against a strong suppressive effect on the systemic Th2 response against OVA.

In our experiments we also found that HK-BCG-induced inhibition of airway eosinophilia was at least to a great extent dependent upon IFN-γ. Furthermore, the lack of eosinophilia in the BCG and HK-BCG treated mice correlated with increased amounts of IFN-γ detected in the BALs of these mice in comparison to the amounts detected in the BALs of PPD or OVA-only treated mice. Since IFN-γ is predominantly produced by Th1 type cells these findings suggest that the Th1 immune response initiated by both live and HK-BCG was responsible for the inhibitory effect on the allergic Th2 response in the lung. Supporting this view is the previously published finding that Th2 cells showed impaired homing into inflamed sites dominated by Th1-type inflammatory reactions [88].

Our results showed that live BCG induced a much stronger inflammatory response in the lung than HK-BCG. Furthermore, it was previously reported that the Th1 immune response

associated with DTH reactions was much lower in mice injected with HK-BCG in comparison to when live BCG was used [54].

Interestingly, as already mentioned above, the application of PPD (derived from *M. tuberculosis*) had no suppressive effect on the development of airway eosinophilia. However, we cannot rule out that using more PPD or applying it more frequently could also reduce the development of airway-eosinophilia. Nevertheless, our results suggest that HK-BCG may contain components other than proteins which were responsible for the inhibitory effect on airway eosinophilia, since the proteins from BCG and *M. tuberculosis* show a strong homology. One potential component of the applied HK-BCG responsible for the inhibition of allergic Th2 responses are CpG oligodeoxynucleotides (CpG-ODN). CpG-ODN are part of the bacterial DNA of BCG (they were initially discovered analysing the immune stimulatory properties of DNA) derived from *M. bovis* [89] and have been shown to suppress the development of allergic asthma in mice [90,91]. Furthermore, it was shown that the application of DNA isolated from BCG inhibited IgE production by human B cells [92]. Further BCG-derived components responsible for the suppressive effect on Th2 responses in the lung may be parts of the cell wall skeleton of BCG (BCG-CWS). These consist of peptidoglycan, arabibogalactan, and mycolic acids. Tsuji et al. reported that HK-BCG and BCG-CWS both induced the maturation of human dendritic cells and more importantly the secretion of IL-12 p40 [89]. Based on this observation, it is likely that the cell wall components of HK-BCG may also induce the production of IL-12 by DC in vivo leading to the inhibition of an allergic Th2 response. IL-12-induced suppression of OVA-induced Th2 responses in the lung has been reported previously [78]. A possible mechanism of how BCG-CWS components may induce IL-12 secretion by macrophages or DC is through the active phagocytosis of these particles. Since IL-12 production by human monocytes was directly linked to the cellular events associated with the phagocytosis of killed *M. tuberculosis* [17].

5.3 Parasitic infections inducing Th2-type responses

A further factor that is believed to be important in the etiology and evolution of atopic disorders is infection with helminths. Our observations, where mice infected with *Nippostrongylus brasiliensis* showed suppression of allergic reactivity, depending on the time of infection, support this hypothesis.

The data available on the possible protective role of parasitic infections in the development of atopic diseases are conflicting. The results of studies that include egg counts suggest that subjects with asthma have a lower parasitic burden than normal subjects. Similarly, African children with urinary schistosomiasis have been reported to have a lower prevalence of atopy than those free of this infection [93].

Although epidemiological studies suggest that asthma is less common where intestinal parasitism is endemic, case-control studies show either no association or an increase in parasitism in people with asthma [94]. Overall, the available data neither refute nor support the theories that parasitic disease either protects against or causes asthma. In contrast to bacterial infections, helminth infection induces very pronounced Th2-type immune responses [73,74]. Therefore, Helminth infections are believed to promote allergic reactions by inducing IL-4 expression and numerous transcription factors enhancing the priming of naive T cells to develop allergen-specific Th2 cells (**Figure 31**). Moreover the increase of eosinophilic inflammation in the airways may lead to a direct trigger of allergic inflammation [60].

A confounding observation is that in developing countries with a high incidence of helminth infections, atopic disorders are not as common as in developed countries [68]. One explanation

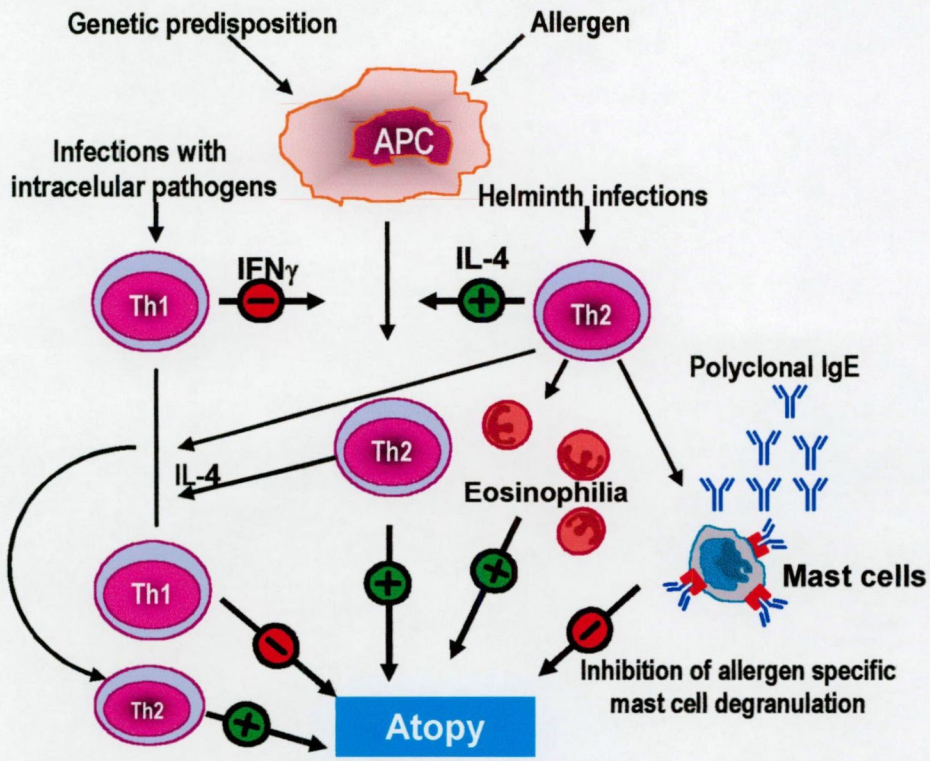

Figure 31. *Model of the possible interactions between infectious diseases and the development of atopic disorders. (1) Genetic and environmental factors determine whether or not allergen-specific Th2 cells are generated; Th2 cells lead to the development of atopic disorders. (2) Infections inducing Th1 responses inhibit the development of allergen-specific Th2 cells by inducing the production of IFN- γ at he site of naive T-cell priming. (3) Established allergic responses or helminth infections deviate Th1 immune responses against pathogens to a mixed Th1/Th2 type by the production of IL-4. (4) Th2 cells specific for the intracellular parasites induce eosinophilia or IgE responses during infection, further exacerbating the development of atopy. (5) Helminth infections directly enhance Th2 responses against allergens by providing IL-4 during allergen-specific Th2-cell development. (6) Increased production and recruitment of eosinophils during worm infections may also contribute to the exacerbation of atopic disorders. (7) The production of polyclonal IgE (induced by metazoic worm infections) competes with allergen specific-IgE binding to mast cells, resulting in the inhibition of mast cell degranulation after allergen challenge. Taken and modified by permission from: Erb K.J. Immunol Today 1999;20:317*

is that humans in developing countries may be exposed more frequently to Th1-inducing bacterial or viral infections resulting in the inhibition of atopic disorders, irrespective of worm infections.

The hypothesis is that although helminth infections might further increase Th2 responses towards allergens, they may also inhibit atopic effector functions [68]. One possible mechanism is that polyclonal IgE induced by worm infections may saturate the Fcε receptors on mast cells and thus competitively inhibit binding of allergen-specific IgE. In this way the allergen-

mediated degranulation of the mast cell, and therefore the development of the allergic cascade, can be suppressed by helminth infection (**Figure 31**). In concordance with the above mentioned facts, our results revealed that a pre-existing helminth infection may potentiate a systemic Th2-type response, yet simultaneously suppress allergen-specific IgE responses and eotaxin production in the lungs depending on the time of infection.

5.4 MHC class II molecule-dependent antigen presentation in Th2 responses depends on the presence of MHC class I molecules

Antigen presenting cells (APCs) such as macrophages, B-cells, and dendritic cells, expresses class II major histocompatibility complex (MHC) molecules when activated. Interactions of MCH II molecules and CD4+ are essential for T cell development and survival in all antigen-specific immune responses [95,96]. Conversely, MHC class I molecules expressed on the surface of APCs are responsible for the recognition of CD8+ cytotoxic T cells. There is virtually no direct relationship between the class I and class II molecule induced antigen processing pathways [97] (**Figure 32**).

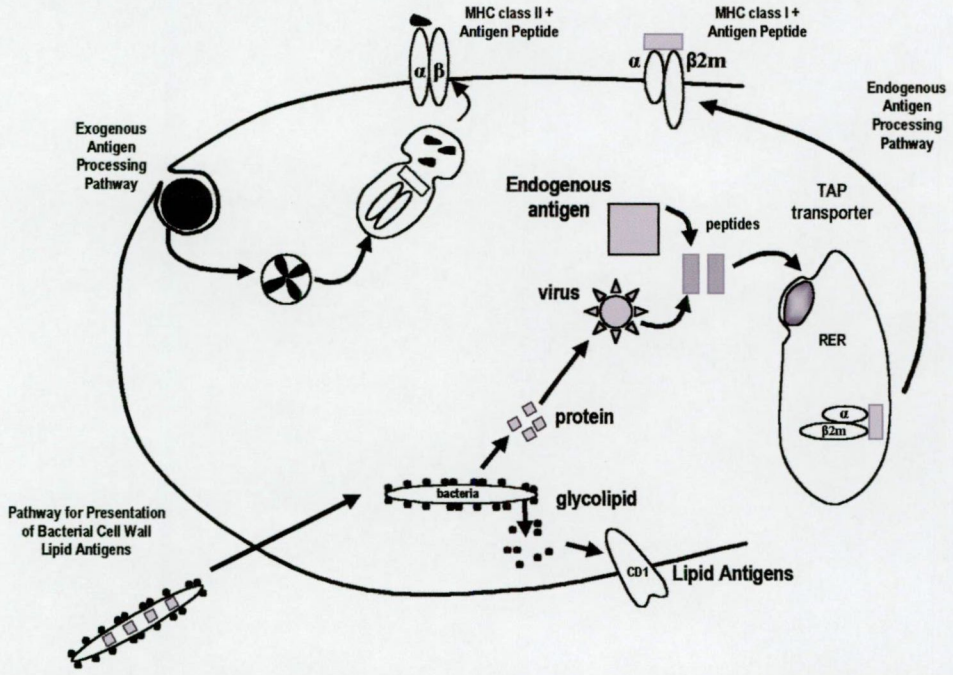

Figure 32. *Antigen processing and presentation pathways. Endogenous MHC class I, exogenous MHC class II, and CD1 antigen presentation pathways are shown.*

Human MHC I mast cells, however, as primary effector cells of the Th2 immune reactions, have been considered to contribute to the induction of an immune response by expressing MHC II molecules [98]. Moreover, MHC I molecules can also serve as superantigen receptors.

Our investigations revealed that MHC class II molecule-dependent antigen presentation in Th2 responses depends on the presence of MHC class I molecules. This finding supports the view that Th1 and Th2-polarized immune responses act interdependently.
Our result shows that the production of the major cytokine responsible for eosinophil recruitment has been inhibited in the absence of MHC I molecules. Taken together, these results support the hypothesis that MHC I molecules play a crucial role in the development of Th2 immune responses [61].

5.5 Influence of infection occurring after atopic sensitization

Th1 inducing infection can inhibit the development of atopy under certain conditions. However, if infections occur when the atopic phenotype have already been fully developed, they are rather more likely to trigger the exacerbation of allergic symptoms than to have a suppressing effect. It appears to depend on the strength of the Th2 response, and thus the amount if IL-4 being produced. The presence of IL-4 during T-cell priming may deviate from the normally dominant Th1 response induced by infection to a mixed Th1/Th2 response, resulting in exacerbation of the atopic disorder [38].

5.5.1 Infection with Influenza A virus leads to Flu antigen-induced cutaneous anaphylaxis in mice (II.)

The results presented in our Flu virus study clearly indicate that the immune response against influenza A virus in BALB/C mice is strongly polarized towards Th1 but also has a minor Th2 component. This can be seen by the large amounts of IFN-γ, the absence of IL-4, and the low levels of IL-5 secreted by the MLN cells after *in vitro* restimulation with Flu-Ag or anti-CD3. The small numbers of eosinophils detected in the lung after infection together with the low titers of flu-specific IgE in the serum of infected mice further supports this view.

Interestingly, we could show that mice infected with flu virus developed virus-specific IgE antibodies and active cutaneous anaphylaxis after intradermal injection with Flu-Ag. Passive cutaneous anaphylaxis could also be demonstrated, suggesting that the detected virus-specific immunoglobulins and not virus-induced alterations of mast cell reactivity were mediating this effect. The passive cutaneous anaphylaxis reaction is a well established method to

demonstrate antibody-mediated mast cell degranulation in the skin [99,100]. It had been shown that after passive transfer of serum both IgE and IgG1 can bind on to mouse mast cells; however, IgG1 antibodies are rapidly cleared from mast cell receptors, whereas IgE antibodies persist for long periods of time. Therefore, IgG1-mediated passive cutaneous anaphylaxis reactions may be detected only 2 h after serum transfer, whereas IgE-mediated mast cell degranulation can be detected 48 h later [99,100]. Our observation that in flu-infected mice specific IgG1 titers were much higher than the specific IgE titers, together with the published finding that local as well as systemic anaphylaxis can occur in the absence of IgE, suggested that IgG1 may be mediating the allergic skin reactivity [101,102]. We could show, however, that passive transfer of IgE depleted flu serum strongly reduced the allergic skin reactivity and that the local anaphylaxis reaction persisted 48h after passive transfer of serum, indicating that mast cell degranulation was mainly mediated by virus-specific IgE. This view is supported by data of *Faquim-Mauro et al.*, showing that IL-4 and IL-12 differentially induce the production of two distinct subtypes of IgG1, which are either inducers or noninducers of passive cutaneous anaphylaxis in mice, respectively [103]. As regards the effect of Flu virus on OVA-induced mast cell degranulation, our observations strongly suggest that virus-specific IgE were mediating allergic skin reactivity. Furthermore, in view of the allergen-specific immunotherapy used in humans, it may be important to note that cutaneous anaphylaxis occurred even though large amounts of virus-specific IgG1 (presumed not to cause mast cell degranulation) [103], IgG2a, and IgG2b were present.

Our finding, that Th1 dominated immune responses such as infections with influenza A virus and the development of Th2 driven allergic responses are not mutually exclusive is underlined by several other studies. It could be shown that respiratory syncytial virus and influenza A virus infection increased ovalbumin-specific IgE production and airways responsiveness in ovalbumin-exposed mice [104,105]. Yamamoto *et al* [106]could show that dendritic cells were associated with the augmentation of antigen sensitization by influenza A virus and that the induced allergen specific immune response was dependent on the timing after flu infection. The enhanced sensitization to aeroallergens after viral infections may partially be due to direct injury of the airways epithelium during an respiratory infections, which may facilitate the entrance of aeroallergens [105,107]. However, viruses have also been reported to stimulate Th2 responses by enhancing mast cell degranulation. *Clementsen et al.* could show *in vitro* that

influenza A virus caused a synergistic enhancement of mediator release from basophils following stimuli such as anti-IgE or antigen [108]. This effect could be abolished by blockade of viral neuraminidase and hemagglutinin [108].

The appearance of virus-specific IgE antibodies could be correlated with croup and wheezing in patients suffering from an infection with the parainfluenza virus [109,110]. In line with other studies our data underlines the importance, that specific IgE antibodies directed against viral or bacterial antigens may be important for infection-associated induction or exacerbation of atopic disease. Although the presence of specific IgE antibodies directed against viral or bacterial substances could be correlated with bronchial hypersensitivity [109,111] little is known about the correlation of virus-specific IgE antibodies and the induction of urticaria or atopic dermatitis.

In concordance with our data, one possibility is that the IgE bound to mast cells may directly cause mast cell degranulation after encountering viral antigen in the skin or the lung during or shortly after the infection.

6 Conclusions

Allergic disorders continue to be a major health hazard in developed countries and no effective preventive measures exist to date. Data are accumulating that implicate a role for decreased incidence of childhood infections in the increasing severity and prevalence of asthma [112]. Animal studies have clearly shown that inducing allergen-specific Th1 immune responses can effectively suppress the development of allergic Th2 responses using different approaches[66,79]. In our experimental mice models we were able to demonstrate some important effects of the major type of pathogens (bacterial, helminth and viral infections) on the development of allergen-induced airway eosinophilia. In the following a short summary of the major conclusions of our experimental work is presented, highlighting the novelties:

6.1 HK-BCG inhibits the development of allergen-induced Th2 responses (I, III)

Our results clearly showed that an intranasal vaccination with live and HK-BCG but not PPD, given 4 or 8 weeks prior to allergen airway challenge, resulted in a strong suppression of airway eosinophilia. We also demonstrated that the inhibition of airway eosinophilia correlated with reduced levels of IL-5 production by T cells from the lymph node of the lungs and a strong reduction in Th2 cell numbers present in the airways of OVA-challenged mice. Furthermore, our findings indicated, that HK-BCG-induced suppression of airway eosinophilia was strongly reduced in IFN-γ deficient mice. Based on these results, in contrast to live BCG, HK-BCG might also be a promising candidate for a prospective asthma vaccine in humans since negative side effects due to mycobacterial infection can be ruled out.

6.2 Th2 immune response induced by helminths is able to suppress or potentiate allergen-induced Th2 response, depending on the time of application

We have established the first animal model exploring the effects of a parasitic infection on allergen-induced systemic immune response. Our results revealed, that Th2 immune response-inducing helminth infections may potentiate allergen-induced, systemic Th2-type reactions, yet simultaneously suppress allergen-specific IgE responses and eotaxin production in the

lungs depending on the time of infection. Our data provide possible explanation to the increase in the prevalence of allergic disorders observed in Westernized societies compared to the lower atopy prevalence in the developing countries, where parasitic infections are endemic.

6.3 MHC class I molecules are necessary for MHC class II molecule-dependent antigen presentation in Th2 responses

Our investigations with MHC class I deficient mice revealed that MHC class II molecule-dependent antigen presentation in Th2 responses depends on the presence of MHC class I molecules. This finding supports the view that Th1 and Th2-polarized immune responses act interdependently.

In addition, this result also supports the hypothesis, that the production of the major cytokine responsible for eosinophil recruitment has been inhibited in the absence of MHC I molecules. In conclusion this is the first animal model, exploring that MHC I molecules play a crucial role in the development of Th2 immune responses [61].

6.4 Flu virus-specific IgE antibodies induce mast cell dergranulation leading to allergic reaction (II)

The Flu virus experiments identified, that infection with influenza A virus induced a strong Th1 response with a minor Th2 component. To the best of our knowledge, this is the first report which provided evidence that virus-specific IgE antibodies can mediate mast cell degranulation leading to allergic skin reactivity in mice. These findings might serve as a new explanation for the mechanism of exacerbation of a pre-existing atopic disease after virus infection.

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9 Original communications