DEVELOPMENT OF VACCINES AGAINST ALLERGIC ASTHMA USING PRODUCTS DERIVED FROM INTRACELLULAR BACTERIA OR HELMINTHS

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In memory of Edison Correa praying for peace and social justice in my country, Colombia...

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1. INTRODUCTION

The Viennese paediatrician Baron Clemens von Pirquet (1874-1929) was the first to introduce the term “allergy” in 1906 from the Greek "allos" meaning changed or altered state and "ergon" meaning reactivity. Von Pirquet used the term to describe the "altered reactivity" he had observed in some diphtheria patients when treated with a horse serum antitoxin. Nowadays, allergies are clearly defined as IgE-mediated immediate hypersensitivity reactions of the immune system to specific substances called allergens (such as pollen, insect venoms, drugs or food). In addition, "atopy" refers to an exaggerated tendency to develop IgE responses to common environmental antigens. The latter term can only be used when IgE sensitization has been documented by IgE antibodies in the serum or by a positive skin prick test (evaluating immediate hypersensitivity reactions after epicutaneous application of the allergen) (Janeway et al., 2001).

Due to the direct interaction with environmental allergens, gastrointestinal and respiratory tract as well as eyes and skin are the places of the body where allergic reactions more often occur. They are manifested in several syndromes including allergic rhinitis (hay fever) or rhinoconjunctivitis, allergic asthma, atopic dermatitis and food allergies. A systemic reaction also occurs when allergens are eaten or introduced directly into the bloodstream as in the case of bee or wasp stings or allergies due to the application of drugs. This entity, called anaphylaxis, is characterized by a sudden increase in vascular permeability and bodywide vasodilatation which can eventually cause a multi-systemic shock rapidly leading to death due to severe hypotension.

Whereas anaphylaxis occurs sporadically, atopic diseases including allergic rhinitis, atopic dermatitis, allergic asthma and gastrointestinal allergies although rarely life-threatening, represent a substantial burden of morbidity and health service cost in the population. Epidemiological data have shown that these diseases afflict more than 20% of the population

in the United States (US) (Strachan et al., 1997). A recent study performed in the United Kingdom (UK) also estimates that 39% of children and 30% of adults have been diagnosed with one or more atopic conditions in this country (Gupta et al., 2004). Moreover, worldwide variations in the prevalence of symptoms of respiratory allergic diseases in the childhood have been reported within the international study of asthma and allergies in childhood (ISAAC). Using a standardized and validated questionnaire, they studied 257.800 children aged 6 to 7 years from 91 centres in 38 countries, and 463.801 children aged 13 to 14 years from 155 centres in 56 countries including Europe, Asia, Africa, Australia, North and South America. The prevalence of rhinitis with itchy-watery eyes (rhinoconjunctivitis) in 1996 varied across centres from 0.8% to 14.9% in the 6-7-year-olds and from 1.4% to 39.7% in the 13-14 year-olds (Strachan et al., 1997). Wheezing prevalence (indicative of asthma) ranged from 4.1 to 32.1% in the younger age group and from 2.1 to 32.2% in the older age group and was particularly high in English speaking countries and Latin America (Asher and ISAAC Steeriing Committee, 1998).

Although a number of medications have been used in the treatment of allergic diseases, most of them are exclusively directed to control the symptoms during the allergic attacks. Only the specific immunotherapy (SIT) offers an alternative to induce long-lasting hyposensitization against the offending allergen. During this therapy, the allergen is repeatedly administered to sensitive individuals in progressively increasing doses over a period of years. Unfortunately, the efficacy of the traditional SIT is limited. While over 90% of patients with hypersensitivity to stinging insects develop clinical tolerance with SIT, only 30 to 50% of allergic rhinitis patients respond, and SIT is even less effective in asthmatics (Horner et al., 2001).

Nevertheless, from all the atopic syndromes, allergic asthma is the one producing most discomfort. Allergic asthma is a common medical problem faced by emergency units and intensive care specialists. Data from Australia, Canada and Spain have reported that allergic asthma accounted for 1 to 12% of all adult emergency visits. Developed economies might

expect 1 to 2% of total health-care expenditures to be spent on asthma. The US studies estimate that the total burden of asthma is approximately $6 billion per year. Direct services (providing health care to asthmatics) represent the greatest part (90%) of the total society cost (Rodrigo et al., 2004).

These data explain the great attention that allergic asthma has received in the last decades. Extensive research has extended from investigation of the basic immune mechanisms leading to atopic asthma to epidemiological studies considering the prevalence of atopy over time and by geographic distribution (McGeady, 2004). All these studies encourage the design of novel asthma vaccines. The development of vaccines along with the improvement of specific therapeutic strategies to resolve asthma symptoms are the two most important branches of research giving hope to decrease the great impact that this disorder is having on health and economy worldwide.

1.1. Immune basis of atopic asthma

Atopic asthma is a chronic inflammatory disorder in which allergen-sensitized individuals develop reversible airway obstruction and airway hyperreactivity (AHR, defined as an increased sensitivity of the airways, as reflected in bronchoconstriction, to several stimuli such as histamine, cholinergic agonists or cold air). It results from the inflammation and spasm of the airways. The inflammation causes a narrowing of the air passages, which limits the flow of air into and out of the lungs. Common symptoms include shortness of breath, wheezing, coughing and chest tightness (Paul, 1997) (Janeway et al., 2001).

After the initial demonstration of the existence of T helper type 1 (Th1, producing IL-2, IFN-γ and TNF-β) and T helper type 2 (Th2, secreting IL-4, IL-5, IL-9 and IL-13) cells in mice and humans (Mosmann et al., 1986) (Romagnani, 1992) many observations have supported the assumption that atopic asthma is the result of Th2-dominated responses to single or multiple

environmental allergens. In asthmatics, this assumption is supported by the increased presence of $CD4^+$ T-cells producing IL-4, IL-5, IL-9 and IL-13 in the airways (Robinson et al., 1992) (Kroegel et al., 1996) (Hauber et al., 2004). The recruitment of Th2 cells is also induced after allergen challenge in patients with allergic asthma (Kroegel et al., 1996). In addition, studies in animal models have further demonstrated the close relationship between allergen-specific Th2 cells and allergic asthma (Taube et al., 2004). Antigen-induced AHR and pulmonary eosinophilia are prevented in T cell-deficient mice or after depletion of murine $CD4^+$ T lymphocytes with specific antibodies (Gonzalo et al., 1996) (Gavett et al., 1994). Furthermore, the application of allergen-specific Th2 cells (but not Th1) generated *in vitro* to naive mice can induce airway eosinophilia, mucus hypersecretion and AHR after allergen challenge (Cohn et al., 1997). However, as summarized in Figure 1, many components of the immune system contribute to the development of allergen-specific Th2 responses. First of all, to recognize the allergen-derived peptides and differentiate into Th2 lymphocytes, $CD4^+$ cells need the help of antigen presenting cells (APC). Although dendritic cells (DC) as well as B cells and macrophages are all professional APC, only DC have the capacity to activate naïve T lymphocytes (Mehta-Damani et al., 1995). In the respiratory tract, an extensive network of DC is located in the mucosa of the nose and large conducting airways of the lung (Schon-Hegrad et al., 1991) (Lambrecht and Hammad, 2003a). In these places, DC have the capacity to capture the allergen (Vermaelen et al., 2001) and then, they become mature and migrate to the draining lymph nodes where the presentation of the allergen to naive $CD4^+$ T cells (in the context of MHC class II molecules) is initiated. Here, DC provide T cells with essential costimulatory molecules and cytokines which finally determine (in the presence of IL-4) the induction of allergen-specific Th2 responses.

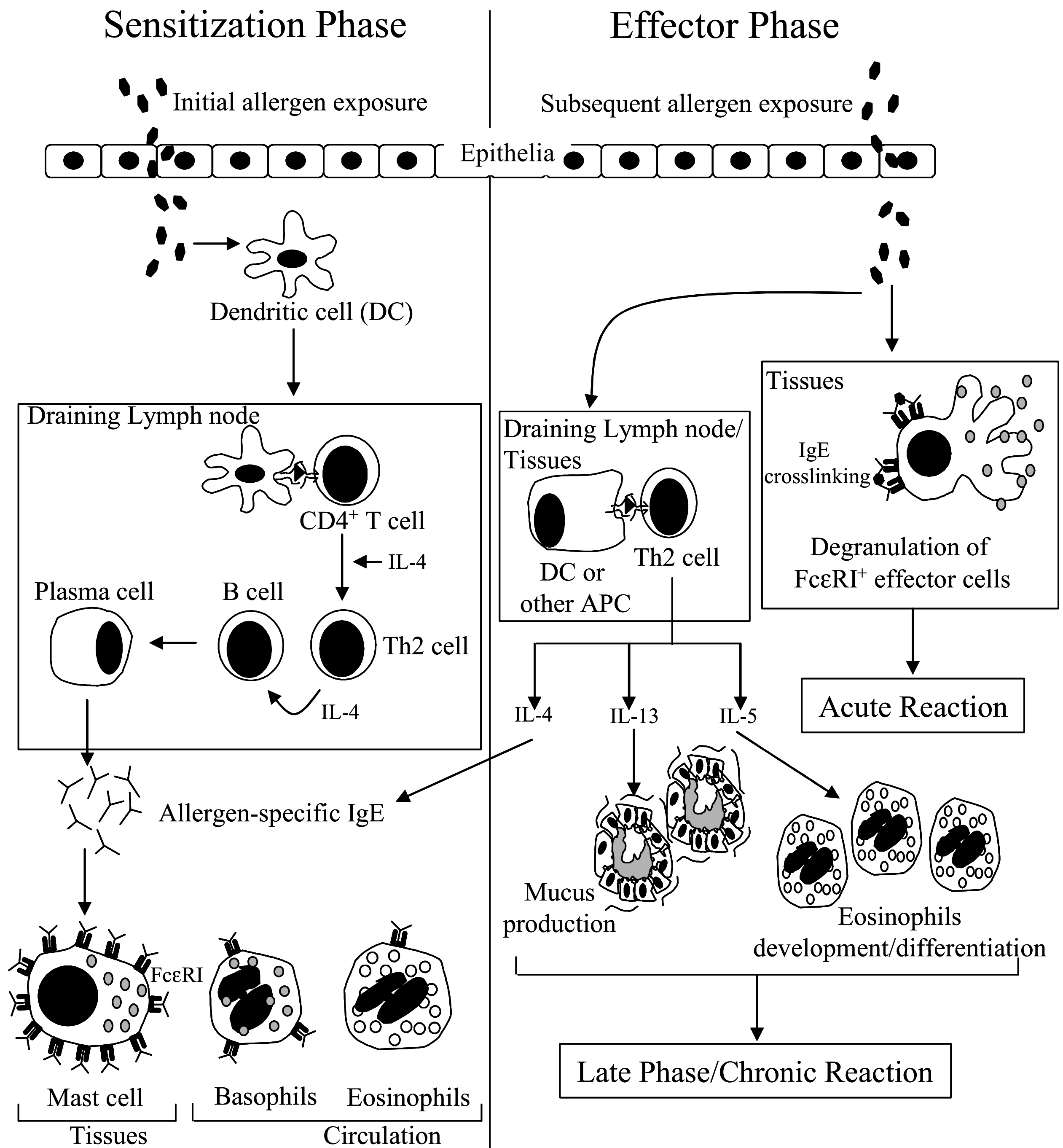

Figure 1. *Immune basis of atopic asthma*

Notably, IL-4, a cytokine essential for Th2-cell development, is not produced by DC. Potential candidates for the early production of IL-4 include a population of NK $1.1^{+}CD4^{+}$ T cells, which can rapidly produce large amounts of IL-4 upon suitable activation *in vivo*, or alternatively, naïve T cell itself in the absence of Th1-skewing signals such as IL-12. Mast cells, basophils and eosinophils have also been suggested to be responsible for this early IL-4 production since they have the capacity to produce this cytokine later during the allergic immune response, however, the initial source of this "early IL-4" is still not well-known (Paul, 1997).

Upon ligation with its receptor, IL-4 activates in T lymphocytes a cytoplasmic protein, the signal transducer and activator of transcription 6 (STAT-6). When activated, this protein dimerizes and traslocates to the nucleus where it modulates transcription through binding to STAT-6-response elements. Among others, STAT-6 induces the expression of the transcription factor GATA-3 (which belongs to a family of transcription factors that bind to a DNA sequence motif, WGATAR (W = A/T; R = A/G)). GATA-3 directly transactivates the IL-5 promoter, inducing IL-5 production. In addition, GATA-3 seems to be a chromatin remodeling factor that allows the transcription of the IL-4/IL-13 locus , playing an essential role in the development of $CD4^{+}$ Th2 cells (Finotto et al., 2004).

Once activated in the draining lymph nodes, Th2 cells provide B cells (located in the germinal centres) with two signals that are crucial for IgE synthesis. First, Th2 cells produce IL-4 in response to antigen receptor engagement. IL-4 induces the transcription of the germline C epsilon gene (corresponding to the constant region of the IgE heavy chain), a process that is further enhanced by CD40 engagement. Cell-to-cell contact between B and T cells through the CD40/CD40 ligand pathway represents the second signal required for the IgE synthesis. In mice, these two signals also enhance the IgG1 class switching in B lymphocytes (Yssel et al., 1998).

IgE (and in mice also IgG1) is essential for immediate hypersensitivity reactions. Most IgE being produced is located predominantly in tissues where it binds to the high-affinity IgE receptor (FcεRI) expressed on the surface of mast cells and basophils (Kawakami and Galli, 2002). Studies in human and mouse mast cells and basophils show a good correlation between the serum IgE concentration and the levels of FcεRI-bound IgE in part because IgE has itself the ability to upregulate the expression of FcεRI. Binding of IgE to FcεRI renders mast cells and basophils "sensitized" to a new challenge with the allergen. Upon secondary exposure with the multivalent allergen, the crosslinking of FcεRI-bound IgE initiates the activation of mast cells and basophils by promoting the aggregation of FcεRI (Kawakami and Galli, 2002).

The role of IL-4 in allergic inflammation is not limited, however, to its capacity to induce the production of allergen-specific IgE/IgG1 by B cells. IL-4 is also able to induce the rolling on and adhesion to endothelial cells of circulating eosinophils (Patel, 1998). These effects are achieved by the capacity of IL-4 to induce the production of eotaxin (a potent chemotactic factor for eosinophils) and to increase the expression of adhesion molecules such as the vascular cell-adhesion molecule-1 (VCAM-1) by endothelial and other structural cells (Patel, 1998) (Mochizuki et al., 1998). In addition, IL-4 acts directly on lung fibroblast inducing a fibrogenic response further amplifying the inflammatory response during the allergic process (Saito et al., 2003).

Other cytokines produced by activated allergen-specific Th2 cells that also contribute to the pathogenesis of allergic asthma are IL-5, IL-13 and IL-9. IL-5 promotes the terminal differentiation of eosinophils from bone marrow precursors and prolongs the viability of mature eosinophils by preventing apoptosis. Moreover, IL-5 selectively enhances the chemotactic responses of eosinophils and upregulates the adhesion of these cells to the endothelium, further increasing eosinophil accumulation. Effector functions of eosinophils such as cytotoxicity or mediator release are also stimulated by IL-5 (Hamelmann and Gelfand, 1999). IL-13, like IL-4, has been shown to contribute to IgE class switching in B cells and has

also the ability to activate lung fibroblasts (Wynn, 2003) (Saito et al., 2003). Moreover, in human respiratory epithelial cells, IL-13 alters mucociliary differentiation and increases the proportion of mucus-positive cells (Laoukili et al., 2001). In addition, human recombinant IL-13, but not IL-4, induces goblet cell differentiation in primary cell cultures from guinea pig tracheal epithelium *in vitro* (Kondo et al., 2002). On the other hand, IL-9 seems to primarily enhance allergic inflammation induced by other Th2 cytokines. It promotes accumulation of lymphocytes and mast cells as well as eosinophilia, enhances IgE production, primes mast cells to respond to allergens and induces mucus secretion by epithelial cells (Hauber et al., 2004).

The skewing of T-cell responses toward the Th2 phenotype with the production of allergen-specific IgE characterizes the "inductor" or "sensitization" phase of the allergic response. Subsequent allergen inhalation in sensitized individuals can elicit three types of responses during the "effector" phase of the allergic process: acute allergic reactions, late-phase reactions and chronic allergic inflammation (see Fig. 1). Acute allergic reactions, which are expressed seconds to minutes after the secondary allergen exposure, reflect the actions of mediators released from mast cells which are normally resident in the tissues at the site of allergen challenge. The crosslinking of FcεRI-bound IgE by multivalent allergens on the surface of mast cells induces the immediate release of preformed bronchoconstrictors, such as histamine and leukotrienes. In mice, mast cells can also be activated to degranulate and release mediators in response to allergen-specific IgG1 dependent signalling through FcγRIII, and this is an additional mechanism to elicit mast cell function during Th2 associated responses (Paul, 1997) (Janeway et al., 2001).

The early asthmatic response is followed, in more than 50% of the subjects, by a delayed bronchoconstrictor response (late-phase reaction) which usually is maximal at 6 to 12 hours after the allergen inhalation and can persist for up to 1 week (Janeway et al., 2001). An important event that triggers the inflammatory cascade during late-phase allergic asthma is the

activation of epithelial, endothelial and smooth muscle cells as well as macrophages and fibroblasts and the subsequent production of chemokines by these cells. Specifically, the production of eotaxins, MCPs (macrophage chemoattractant proteins), MDC (macrophage-derivated chemokine) and RANTES acting in a coordinated co-operative manner, direct the migration and recruitment of allergen-specific Th2 cells, basophils and eosinophils (expressing the chemokine-receptors CCR2, CCR4 and most importantly CCR3) to the sites where the allergen challenge takes place (Gutierrez-Ramos et al., 2000). Basophils represent an important source of inflammatory mediators and cytokines after IgE-dependent activation and degranulation (Kawakami and Galli, 2002). In addition, eosinophils are considered to be the central pro-inflammatory leukocytes involved in the asthmatic reaction, due in part to the secretion of toxic granular proteins and membrane products that induce pulmonary damage and subsequently intensify AHR (Janeway et al., 2001). Interestingly, it seems that IgG and/or cytokines, such as IL-5, are more important than IgE for eosinophil activation. This assumption is supported by studies indicating that eosinophils do not exert effector functions after IgE-dependent stimulation although they express low levels of FcεRI on the cell surface (Kita et al., 1999).

Chronic allergic inflammation typically occurs at anatomic sites that have been repeatedly challenged with allergen over prolonged periods. Sites of chronic allergic inflammation not only contain effector cells that have been recruited from the circulation such as eosinophils and Th2 cells, but also are often associated with alterations in structural cells and tissues in the asthmatic airways. This process has been collectively named airway remodelling and includes events such as wall thickening, subepithelial fibrosis, mucus metaplasia and myofibroblast hyperplasia and hypertrophy. It seems that the prolonged exposure to Th2-derived cytokines favours the development of remodelling upon which airways become characteristically hyperreactive. During this phase of the disease, factors other than re-

exposure to the allergen (e.g. irritants, virus infections) can trigger asthma attacks (Elias et al., 1999).

1.2. Factors influencing the development of allergic Th2 responses

Although the immunological processes leading to the development of allergic diseases are relatively well defined, it is still not understood why the exposure to allergens causes atopic disorders in some individuals but not in others. However, it is clear that both genetic as well as environmental factors are involved. Concordance rates for the occurrence of allergic diseases are higher in monozygotic twins than in dizygotic twins indicating that genetic factors play an important role in the development of allergic disorders. However, genetically identical twins are often discordant in their expression of atopy implying heritability of only 60 to 73% (Paul, 1997) (Skadhauge et al., 1999). Similarly, individuals with two atopic parents are at greater risk of developing an allergic disease than those with only one atopic parent (approximately 47% versus 24%), but the specific allergic diseases in those individuals may be different from those of the parents (Paul, 1997). Overall, results of the genome screens for asthma-related traits in 11 different populations provide compelling evidence for at least 18 genes contributing significantly to the development of asthma and atopy. Among others, genes involved in the IL-4/IL-13 pathway have been associated with atopy in many studies (Hoffjan and Ober, 2002).

Although the role of heritability in the development of atopic diseases is unquestionable, it is not able to explain the "epidemy of atopy" that has been observed over the past three decades. The term "epidemy of atopy" refers to a marked increase in the incidence of atopic disorders within a few decades reported especially in developed as opposed to developing countries. Recent data from a questionnaire survey performed in Aberdeen, Scotland show that the reported diagnosis of asthma in this area rose from 4.1% in 1964 to 24.0% in 1999 (Devenny et al., 2004). Similar reports have been published by other European countries and the United

States (Aberg et al., 1995) (Hartert and Jr, 2000) (Akinbami and Schoendorf, 2002). The reason for this phenomenon is still not known although there are several explanations that could account for it. It is possible that the genetic background of the human population living in developed versus developing countries is different, resulting in a higher propensity to develop atopic diseases. However, this appears not to be the case, as people from developing countries show an increase in allergic diseases when they are exposed to western living conditions (Waite et al., 1980). Thus, the exposure to western living conditions or "westernization" seems to be more associated with the "epidemy of atopy" than the genetic background of the population.

Westernization refers to all changes established in affluent western countries over the past 50 years, including the arrival of new environmental irritants due to industrial pollution as well as the improvement in public health and hygiene programs, limiting the exposure to infectious agents. Interestingly, the increase in atopic disorders observed in former East Germany has been partly attributed to an increase in westernization. These phenomenon could be explained by the increase in industrial pollution levels and consecuently, higher atopy rates. Contrary to preliminary expectations, German studies have shown that the prevalence of atopic conditions is not related with the exposure to polluted environments (Hirsch et al., 1999). Comprehensive studies performed in the UK also conclude that it is unlikely that air pollution has contributed substantially to the rise in prevalence of asthma and allergic disease in recent decades (Devereux et al., 1996).

Finally, it has been speculated that the increase in atopy is due, in part, to the steady decline of infectious diseases in the developed world, a phenomenon designated by Strachan in 1989 as the "hygiene hypothesis" (Strachan, 1989). According to this theory, the exposure to infectious agents in the early childhood prevents the development of allergen-specific Th2 cells because they establish Th1-based immunity (McGeady, 2004) (Hertzen and Haahtela, 2004). This assumption is supported by previous studies indicating that Th1 cells producing

cytokines such as IFN-γ are able to suppress Th2 immune responses both *in vitro* and *in vivo* (Gajewski and Fitch, 1988) (Parronchi et al., 1992) (Lack et al., 1996) (Li et al., 1996).

Indeed, some features of the immune response early in life strongly support the hygiene hypothesis. During pregnancy there is a bias towards Th2-type immune responses in order to antagonize Th1 responses that could otherwise lead to fetus rejection. This tendency is maintained in the newborn which presents abundant Th2 cytokines and transiently produce IgE antibodies (McGeady, 2004). In most of the infants, however, this tendency is lost presumably due to the exposure to viral and bacterial pathogens or commensales, which leads to an environment rich in IFN-γ. Limited exposure to these organisms during early childhood (as result of westernization) leads to insufficient stimulation of Th1 cells, which in turn cannot counterbalance the expansion of Th2 cells and results in a predisposition to allergy. This implies that non-atopic individuals should also recognize harmless environmental allergens but the bias toward Th2-type responses does not occur. Indeed, studies on immune responses to allergens in healthy individuals demonstrate that a peripheral T cell repertoire to allergens exists that recognizes the same epitopes as allergic patients (Ebner et al., 1995). Moreover, another report shows that peripheral blood T cells from 100% of adults and 60% of infants (atopic and nonatopic) exhibited vigorous proliferative responses when stimulated with ubiquitous inhalant allergens reinforcing the notion that qualitative (i.e. Th1 vs Th2) more than quantitative variations in specific T cell reactivity ultimately determine the allergen responder phenotype (Upham et al., 1995).

1.3. Evolution of the hygiene hypothesis: from effector to regulatory immune responses

Substantial body of evidence from epidemiological studies appear to provide support to the hygiene hypothesis. A cohort study performed in Guinea-Bissau, demonstrated that measles infection was associated with a large reduction in the risk of skin-prick test positivity to house dust mites in African children (Shaheen et al., 1996). Data from Matricardi et al., showed that

respiratory allergy was less frequent in people heavily exposed to orofecal and foodborne microbes such as *Toxoplasma gondii*, Hepatitis A virus and *Helicobacter pylori* in Italy (Matricardi et al., 2000). Similar results were obtained examining a public database from a cross-sectional survey in the US (Matricardi et al., 2002). Moreover, the exposure to mycobacteria species, potent inducers of Th1 immune responses, has been also strongly associated with a decrease in the appearance of allergic disorders. Shirakawa et al., found that a positive tuberculin test (indicative of a specific cellular immune response against mycobacteria) inversely correlated with the subsequent development of atopy and asthma in 12- to 13-year-old schoolchildren vaccinated with BCG (Bacillus Calmette-Guerin) in Japan (Shirakawa et al., 1997). BCG is an attenuated strain of *Mycobacterium bovis* widely used worldwide to prevent tuberculosis in childhood. Furthermore, it was also reported that BCG vaccination early in infancy prevented the development of atopy in African children (Aaby et al., 2000). A multicentre study including 23 countries in Europe as well as the US, Canada, Australia and New Zealand demonstrated that the increase in tuberculosis notification rates was inversely associated with the prevalence of wheezing, asthma and allergic rhinoconjunctivitis (Mutius et al., 2000). A lower prevalence of asthma was also found in Finnish women who had tuberculosis before the age of 20 (Hertzen et al., 1999). However, it is important to emphasize that the influence of mycobacterial exposure on the suppression of allergic disorders may vary depending on the genetic background of the population and in the case of BCG, on the age of vaccination and the BCG strain and doses applied. Probably these are the reasons why other studies have failed to find a correlation between BCG vaccination and development of allergy (Alm et al., 1997) (Yilmaz et al., 2000) (Omenaas et al., 2000) (Wong et al., 2001) (Anderson et al., 2001) (Jang and Son, 2002) (Jentoft et al., 2002) (Bager et al., 2003).

Additionally, epidemiological studies have also shown that not only infections caused by bacterial and viral pathogens but also the exposure to Th1-inducing bacterial components

could decrease the development of allergic diseases. Recent reports indicate that indoor endotoxin exposure early in life may protect against allergic sensitization (Braun-Fahrlander et al., 2002) (Eder and Mutius, 2004). Endotoxin (also referred to as lipopolysaccharide (LPS)) is a conserved bacterial component and one of the most well known activators of the innate immune system. This molecule is an integral constituent of the outer membrane of gram-negative bacteria. When peripheral immature DC are exposed to LPS, the maturation of these APC is induced, increasing the expression of MHC class II and costimulatory molecules and stimulating the production of proinflammatory cytokines such as TNF-α, IL-1 and IL-6. Moreover, LPS induces the production of IL-12 by these cells, promoting the generation of Th1 immune responses (Hilkens et al., 1997). These immunomodulatory properties of LPS further support epidemiological findings suggesting an influence of LPS exposure on the development and maintenance of allergic diseases.

The data presented so far support a hygiene hypothesis scenario on which a scarce Th1 stimulation due to reduced microbial contact at an early age results in an increased sensitivity to develop Th2-mediated allergic responses. However, epidemiological observations demonstrate that the occurrence of autoimmune diseases has risen simultaneously with the increase in the prevalence of atopic asthma in developed countries (Sequeira et al., 1993) (Bach, 2001) (Stene and Nafstad, 2001) (Sheikh et al., 2003). Higher exposure to infectious agents appears also to confer protection against the development of autoimmune diseases (David et al., 2004). Since these are Th1-mediated disorders, this cannot merely be explained by a Th1/Th2 imbalance. Similar concerns arise when considering that helminth infections, which are strong inducers of Th2 immune responses, are at least as prevalent as Th1-inducing infectious diseases in developing countries. Therefore, worm infections would be expected to promote atopy through the induction of IL-4 production leading to an increased development of Th2 cells. Interestingly, this phenomenon was only observed when the individuals had relatively low parasite loads. Heavy and chronic helminth infections (i.e. with *Schistosoma sp.*

or intestinal helminths) have been inversely associated with the development of allergy (Araujo et al., 2000) (Nyan et al., 2001). Moreover, long-term treatment against intestinal helminths increased atopic reactivity in heavily parasitized subjects (Lynch et al., 1993). An interesting explanation for these results was obtained from a study performed in an endemic area for *Schistosoma haematobium* infection in Africa (Biggelaar et al., 2000). This survey showed that harbouring *S. haematobium* infection decreased the chance of a positive skin reaction to an allergen extract by 68%. High production of IL-10 by peripheral blood mononuclear cells in response to adult worm antigen of *S. haematobium* significantly correlated with a lower risk of a positive skin reaction to the allergen. The authors concluded that the anti-inflammatory cytokine IL-10, induced during chronic schistosomiasis, is central to suppress atopy in African children. IL-10 was first described as an inhibiting factor secreted by Th2 cells which was able to suppress the proliferation of Th1 cells. However, in recent years it has become apparent that T regulatory 1 (Tr1) cells can produce high levels of this cytokine and have the capacity to inhibit Th2 immune responses *in vitro* as well as allergic asthma *in vivo* (Cotters et al., 2000) (Robinson et al., 2004). Thus, it seems reasonable that chronic helminth infections inducing the generation of Tr1 cells as part of an immunregulatory mechanism to prevent harmful T cell responses, may in turn, reduce the susceptibility to develop atopic diseases. In fact, recent studies showed that allergen-specific IL-10-secreting T cells are the predominant subset found in healthy individuals in comparison with allergic patients (Bullens et al., 2004) (Akdis et al., 2004).

These findings describe how the initial hygiene hypothesis evolved from a pure Th1/Th2 imbalance to an extended scenario on which immunregulation plays an important role (see Fig.2). Indeed, it appears that not only helminths but also a variety of other microorganisms induce the production of IL-10 and TGF-β by regulatory T (Tr) cells thus alleviating airway inflammation and promoting tolerance to respiratory allergens. *Mycobacterium vaccae*, a rapid growing soil organism, appears to induce $CD4^{+}CD45RB^{lo}$ regulatory T cells, which

inhibit the development of airway eosinophilia. This specific inhibition is mediated through IL-10 and TGF-β (Zuany-Amorim et al., 2002). Moreover, commensal gut microflora has been reported to suppress allergic Th2 responses. These bacteria, besides of inducing the production of IL-12, also stimulate the secretion of TGF-β thereby decreasing the sensitivity to inhaled allergens (Rautava et al., 2002).

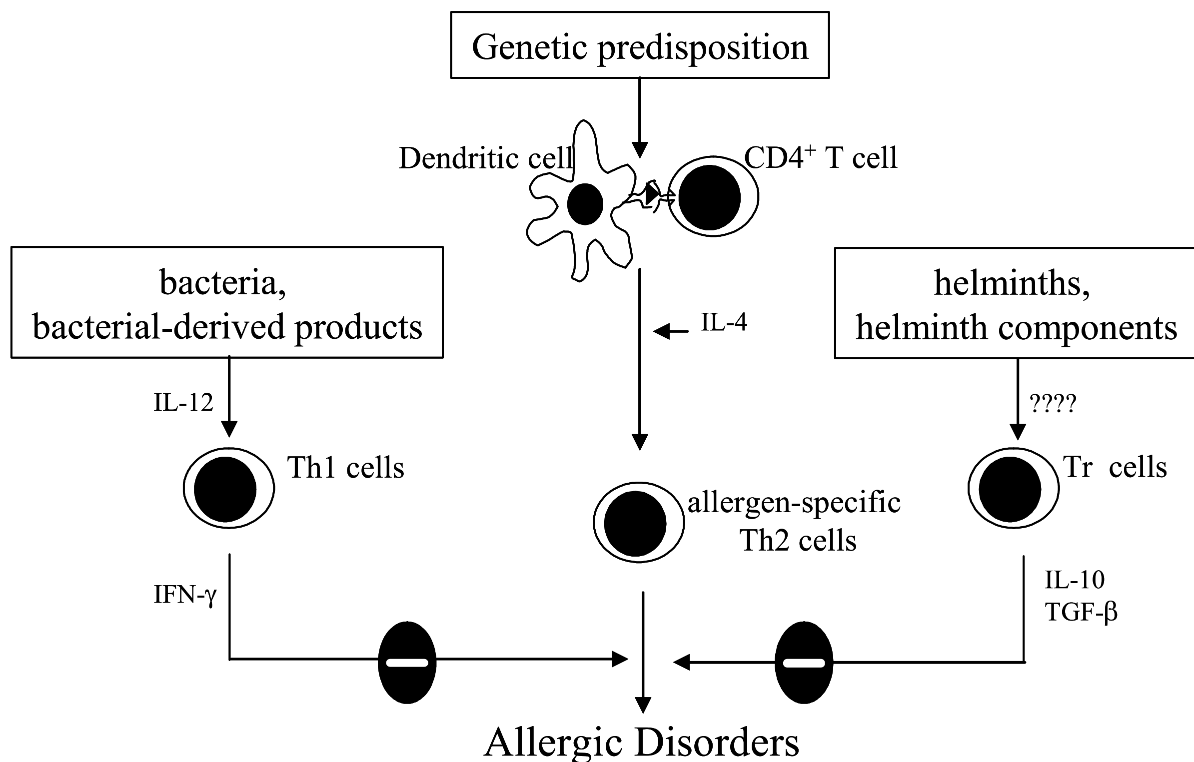

Figure 2. *Immune basis of the hygiene hypothesis*

Additional evidence about the role of Tr cells in the control of asthma symptoms was obtained from the evaluation of patients submitted to SIT. When effective, SIT modifies mucosal Th2 responses in favour of Th1 responses and increases IL-10 production in peripheral and mucosal surfaces. These IL-10-producing cells appear to be identified by the $CD4^{+}CD25^{+}$ phenotype (Robinson et al., 2004).

1.4. Th1-inducing adjuvants as promising candidates for the development of anti-allergy vaccines

Based on the epidemiological studies, it can be suggested that Th1-inducing adjuvants could be candidates for the development of anti-allergy vaccines. This possibility has been evaluated in different animal models. Numerous studies have demonstrated that infection with *M. bovis*-BCG prevents airway eosinophilia, the development of AHR and induces a partial reduction in the levels of allergen-specific IgE/IgG1 serum antibodies in the model of ovalbumin (OVA)-induced allergic disease in mice, rats or guinea pigs (Erb et al., 1998) (Koh et al., 2001) (Su et al., 2001) (Hopfenspirger and Agrawal, 2002). A reduction in the levels of Th2 cytokines detected in draining lymph nodes and spleen cell cultures or bronchoalveolar lavages (BAL) is frequently associated with these findings. The suppressive effect of BCG on the development of allergic Th2 responses seems to be very strong since vaccination with this product 16 weeks prior to OVA airway challenge is able to inhibit bronchial eosinophilia, AHR and reduced IgE levels even in newborn Th2-susceptible hyper-IgE mice (Nahori et al., 2001). An additional study also shows decreased levels of total serum IgE in Th2-predisposed Brown Norway rats treated with live BCG during the OVA sensitization period (Hylkema et al., 2002). Interestingly, it appears that bacteria do not have to be alive to prevent allergic responses, since killed BCG also shows a protective effect (Major et al., 2002). Moreover, intranasal application of purified protein derivative of *Mycobacterium tuberculosis* (PPD) is able to prevent the development of allergic rhinitis in mice (Hattori et al., 2002). More recently, it was reported that mycobacterial chaperonins and lipoglycans including the mycobacterial cell wall component ManLAM (mannose-capped lipoarabinomannan) have also the ability to inhibit allergen-induced pulmonary eosinophilia in the murine model of allergic airway disease (Riffo-Vazquez et al., 2004) (Sayers et al., 2004).

The results from the experimental studies have encouraged the evaluation of BCG as a treatment for asthma in humans. Clinical trials showed that cutaneous injection of BCG

significantly increased forced expiratory volume, forced expiratory flow in the lung and reduced medication in adults with moderate-to-severe asthma. These effects were accompanied by suppressed Th2-type immune responses (Choi and Koh, 2002). Another study reported the reduction in total and allergen-specific IgE levels after BCG vaccination in volunteers with a history of allergic rhinitis (Cavallo et al., 2002).

Taken together, these studies suggest that mycobacteria or mycobacterial products may be useful for the design of vaccines or therapies aimed at protecting against allergic diseases. The induction of specific Th1 responses appears to be the major mechanism involved in the BCG-induced inhibition of allergic Th2 responses since the anti-allergic effect of BCG is strongly reduced in IFN-γ- or IFN-γ receptor-deficient mice (Erb et al., 1998) (Major et al., 2002). Moreover, several studies have shown increased levels of IFN-γ after *in vitro* stimulation of lung lymph node or splenic cells from BCG-vaccinated animals subsequently immunized with OVA (Nahori et al., 2001) (Koh et al., 2001) (Yang et al., 2002). These findings are often accompanied by elevated anti-OVA IgG2a serum levels, which are stimulated by IFN-γ, suggesting that antigen-specific Th1 responses were generated (Su et al., 2001). It has been speculated that one of the major reasons why mycobacteria can induce potent Th1 responses is that they express specific molecules referred to as pathogen-associated molecular patterns (PAMPs) that are recognized by host cells. PAMPs are widely conserved among different bacterial species and play an important role in the activation of both innate and adaptive immune responses. Most but not all PAMPs bind to Toll-like receptors (TLR) expressed by a wide variety of immune cells leading to cell activation (Takeda and Akira, 2005). BCG-mediated TLR activation may participate in IFN-γ induction for lymphocytes through IL-12 modulation in APC. TLR-2 and TLR-4 have been implicated in this process via interaction with BCG-cell wall skeleton components which consist of mycolic acids, arabinogalactan and peptidoglycan (Seya et al., 2002).

Another prominent example of PAMPs are the CpG-oligodeoxynucleotides (ODN). CpG-ODN are non-coding, non-methylated DNA sequences with central C-G dinucleotides commonly found in bacterial DNA and first described in mycobacterial extracts (Tokunaga et al., 1992). Viruses and eukaryotic organisms also contain CpG motifs, however, to a lower degree and they do not activate eukaryotic cells because they are methylated. Bacterial CpG-ODN have been found to exert multiple stimulatory effects on immune cells including DC, macrophages, B cells, NK and T cells (Krieg, 2002). They are potent mitogens and improve the antigen-presenting capacity of DC and macrophages through the upregulation of costimulatory molecules and the induction of proinflammatory cytokines. In addition, CpG-ODN are potent stimulators of IL-12 and IFN-γ production by APC or NK cells, respectively. CpG-ODN lead to the activation of these cells through the binding of another Toll-like receptor, TLR-9 (Krieg, 2002).

A special feature of CpG-ODN-induced immune responses is that they are also strongly biased towards Th1. For this reason, CpG-ODN have been used as adjuvants in vaccines designed to protect against the development of allergic diseases. Several studies in experimental animal models have shown that the application of CpG-ODN can down-regulate allergen-induced Th2 responses (Kline et al., 1998) (Shirota et al., 2000) (Serebrisky et al., 2000) (Banerjee et al., 2004). The basis of this inhibition seems to be an immune deviation from an allergen-specific Th2 to a Th1 response (Wohlleben and Erb, 2001). Controversially, Kline et al. reported that IFN-γ and IL-12 are not needed for CpG-ODN-mediated inhibition of allergic responses. However, in the absence of IFN-γ, mice require 10 times as much CpG-ODN to be protected against the induction of airway eosinophilia (Kline et al., 1999).

In addition to mycobacteria and CpG-ODN, other strategies that bias the immune response to the Th1 phenotype, have shown to reduce allergic responses. For example, the application of live or heat-killed *Listeria monocytogenes* have been reported to suppress OVA-induced Th2 responses (Mizuki et al., 2001). It seems that this bacteria inhibits the development of allergic

responses in mice by inducing the production of IL-12/IL-18 leading to the generation of $CD8^+$ T cells secreting IFN-γ (Yeung et al., 1998) (Hansen et al., 2000). On the other hand, it has been demonstrated that plasmid gene vaccination utilizing allergen expression vectors, highly stimulates Th1-biased immune responses, also preventing the development of Th2-biased immunity (Horner et al., 2001).

Taken together, the results from these studies support the idea that vaccines aimed to induce allergen-specific Th1 responses could be used as candidates to prevent allergic diseases. However, it should be pointed out that Th1 cells are effector cells with a potent pro-inflammatory capacity. Experiments using monoclonal TCR transgenic Th1 cells recognizing specific OVA peptides indicate that OVA-specific Th1 cells can cause lung neutrophilia and inflammation after OVA intranasal challenge (Hansen et al., 1999) (Randolph et al., 1999) (Takaoka et al., 2001). Importantly, the influx of neutrophils into the airways has been particularly associated with the remodeling process in asthma (Sun and Chu, 2004). Further danger associated with vaccines that induce Th1 responses may be toxic shock as reported for the use of high amounts of CpG-ODN (Sparwasser et al., 1997). In addition, other evidences suggest that the production of large amounts of pro-inflammatory cytokines induced by Th1 vaccines might lead to a breaking of immunological tolerance needed to inhibit the development of autoimmune disorders (Erb and Wohlleben, 2002). For this reason, it is important to emphasize that, in order to use Th1-inducing adjuvants for an anti-allergy vaccination, potential harmful side effects need to be carefully monitored. Also, studies comparing the efficacy and safety of the different Th1-inducing adjuvants should be conducted in order to select the most appropriate vaccine candidate to be evaluated in human clinical trials.

1.5. DC-based strategies to induce allergen-specific Th1 immune responses

As already mentioned, vaccination with live or dead bacteria, bacterial components, CpG-ODN or plasmid DNA suppresses the development of allergen-specific Th2 responses mainly because these products induce potent Th1 immune responses. Interestingly, the Th1-promoting ability of these products seems to be associated with their capacity to induce the production of IL-12 by DC. In addition, the increased presence of DC in the airways of atopic asthmatic individuals and allergen-exposed animals suggest that they critically contribute to the pathogenesis of atopic asthma (Lambrecht and Hammad, 2003b). Experimental evidence also indicates that depletion of airway DC during secondary exposure to inhaled allergen induce the disappearance of eosinophilic airway inflammation and goblet cell metaplasia (Lambrecht et al., 1998). Furthermore, the application of allergen-pulsed myeloid DC into the airways is able to induce allergic inflammation after challenge with the specific allergen (Lambrecht et al., 2000) (Sung et al., 2001) (Graffi et al., 2002). Based on these findings, it is possible that targeting the function of airway DC it might be possible to prevent the development of asthma. Animals vaccinated with DC pulsed with specific antigens have shown very efficient protection against infectious diseases or cancer (Berger and Schultz, 2003). More recently, bone marrow-derived DC (BM-DC) maturated with CpG-ODN in the presence of *Leishmania major*-lysate were shown to be able to reduce the footpad swelling and parasitic load in susceptible mice infected with *L. major* (Ramirez-Pineda et al., 2004). In this study, the protection was mediated by the induction of specific Th1 responses. Another report shows that retrovirally mediated overexpression of IL-12 in DCs strongly polarized naive OVA-specific $CD4^+$ T cells toward Th1 effector cells *in vitro*. After intratracheal injection, OVA-pulsed IL-12-overexpressing DCs failed to induce Th2 responses *in vivo* and no longer primed mice for Th2-dependent eosinophilic airway inflammation upon OVA aerosol challenge (Kuipers et al., 2004). These reports encourage the evaluation of DC-based strategies as candidates for the development of anti-allergy vaccines. The maturation of

allergen-loaded DC in the presence of a strong Th1-inducing adjuvant may stimulate the production of IL-12 by these cells, which in turn, once applied into naïve recipients, would induce the generation of allergen-specific protective Th1 immune responses. Experimental studies should be designed in order to evaluate this approach as a way to prevent the deleterious effects of allergic Th2 responses.

1.6. Helminth-derived components may also prevent allergic Th2 responses

As previously described, besides bacteria or bacterial-derived products, circumstantial evidence in humans suggests that helminth infections can modulate the generation of Th2 immune responses. Moreover, animal studies have further supported these observations. The first report on this area was obtained from experiments with *Strongyloides stercoralis* infection in mice (Wang et al., 2001). When *S. stercoralis*-infected animals were subsequently immunized and challenge intratracheally with OVA, the authors observed that the pre-existing parasite infection enhances Th2-related cytokines in the airways. Despite of this finding, parasite-infected animals did not exhibit higher levels of airway eosinophilia than uninfected animals after the OVA-immunization and challenge. In addition, the parasite infection induced a decrease in the levels of eotaxin and OVA-specific IgE in the airways. Moreover, other studies have shown that the infection with the helminth *Heligmosomoides polygyrus* greatly diminished allergen-specific IgE levels in allergen-sensitized mice (Bashir et al., 2002). This effect was accompanied with reduced symptoms of systemic anaphylaxis. Strikingly, treatment of infected mice with neutralizing antibodies to IL-10 abrogates infection-mediated protection of allergic responses. Additional evidence of the involvement of IL-10 in the helminth-mediated suppression of allergen-induced airway inflammation was obtained from a study with *Nippostrongylus brasiliensis* infection in mice (Wohlleben et al., 2004). During its pulmonar-enteric lifecycle, *N. brasiliensis* worms induce strong Th2 responses characterized by eosinophilia and the secretion of IL-4, IL-5 and IL-10 which are

responsible for the elimination of the parasite (Lawrence et al., 1996). In the murine model of OVA-induced Th2 responses, *N. brasiliensis* infection decreases the allergen-induced eosinophilia and eotaxin levels in the airways. These effects correlated with a reduction in the OVA-specific IgG1 and IgE levels in the BAL. Interestingly, the *N. brasiliensis*-mediated suppression of the allergen-induced Th2 responses was not observed in IL-10-deficient mice. Taken together, these studies suggest that helminths produce substances which can effectively downregulate allergic responses likely by inducing immunregulation. Some advances have been obtained in this area. For example, adult worm extracts of *Ascaris suum* were found to alleviate allergic symptoms and allergen-induced Th2 responses in mice although the mechanism was not described (Lima et al., 2002). In another study, nippocystatin was identified as a new cysteine protease inhibitor derived from *N. brasiliensis* excretory-secretory products (NES) (Dainichi et al., 2001). Although NES had been previously reported to act as an adjuvant for the generation of Th2 responses (Holland et al., 2000) (Balic et al., 2004) (Holland et al., 2005), NES-derived nippocystatin was able to profoundly suppress OVA-specific proliferation of splenocytes in OVA-immunized mice. OVA-specific cytokine production (IL-4 as well as IFN-γ) and IgE levels were also greatly downregulated by the intraperitoneal application of this product (Dainichi et al., 2001). In addition, a schistosoma-specific phosphatidylserine that influences the development of IL-10-producing Tr cells acting through the ligation of TLR-2 in DC has also been identified (Kleij et al., 2002). Overall, all these finding indicate that helminth-derived components may be useful in supressing allergic responses. New helminth-derived components need to be evaluated and further investigations are necessary in order to clearly elucidate the mechanisms by which these products can ameliorate allergic symptoms in the presence of a strong Th2 environment.

2. AIMS OF THE THESIS

The increasing scientific evidence supporting the hygiene hypothesis provides a solid basis for the development of new anti-allergy vaccines. In this thesis, three different strategies were tested in order to identify promising vaccine candidates against allergic asthma in the murine model:

1. Compare the efficacy of four different known Th1-inducing adjuvants, i.e. live BCG, heat-killed BCG, CpG-ODN and PPD, as components of vaccines aimed at inhibiting allergic asthma.

2. Design a dendritic cell-based vaccination strategy leading to the induction of allergen-specific Th1 cells and then, investigate whether it protects mice from developing allergen-specific Th2 responses.

3. Evaluate NES, a product derived from the rodent helminth *N. brasiliensis*, as a new potential adjuvant to prevent the development of allergic responses.

3. MATERIALS AND METHODS

3.1. Mice

Female BALB/c or C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany) and were maintained under conventional conditions in an isolation facility. IFN-γ-, IL-12p35- and IL-12p35/p40-deficient mice (all on a C57BL/6 genetic background) were generously provided by O. Liesenfeld (Berlin, Germany). IL-10-deficient mice (on a C57BL/6 genetic background) were a gift of W. Müller (Cologne, Germany). TLR-4-deficient mice (on a C3H/HeJ genetic background) were purchased also from Charles River and TLR-2/TLR-4-double deficient mice (on a C3H/HeJ genetic background) or MyD88-deficient mice (on a C57BL/6 genetic background) were a gift of C. Kirschning (München, Germany). Mice transgenic for the $OVA_{323-329}$-specific αβTCR (OT-2) (on a C57BL/6 genetic background) were kindly provided by A. Schimpl (Würzburg, Germany). At the onset of the experiments, animals were between 6 and 8 weeks of age. All experiments were performed according to the guidelines of the local and government authorities for the care and use of experimental animals.

3.2. Induction of OVA-specific Th2 responses

In order to induce OVA-specific Th2 responses, mice were treated as described in Figure 3. Briefly, a mixture of 2 μg OVA (Sigma Chemical Co., St. Louis, MO) in 200 μl alum (a strong Th2-inducing adjuvant) (Serva, Heidelberg, Germany) was applied twice intraperitoneally (i.p.) (day 0 and 14) to the mice in order to prime OVA-specific Th2 cells. Subsequently, mice were anaesthetized by an i.p. injection of Ketamine/Xylazine (Sigma) and challenged intranasally (i.n.) with 50 μl PBS containing 100 μg OVA (day 24) to induce the allergic symptoms in the lung and airways. Analyses were performed 6 days after the i.n. challenge unless otherwise indicated.

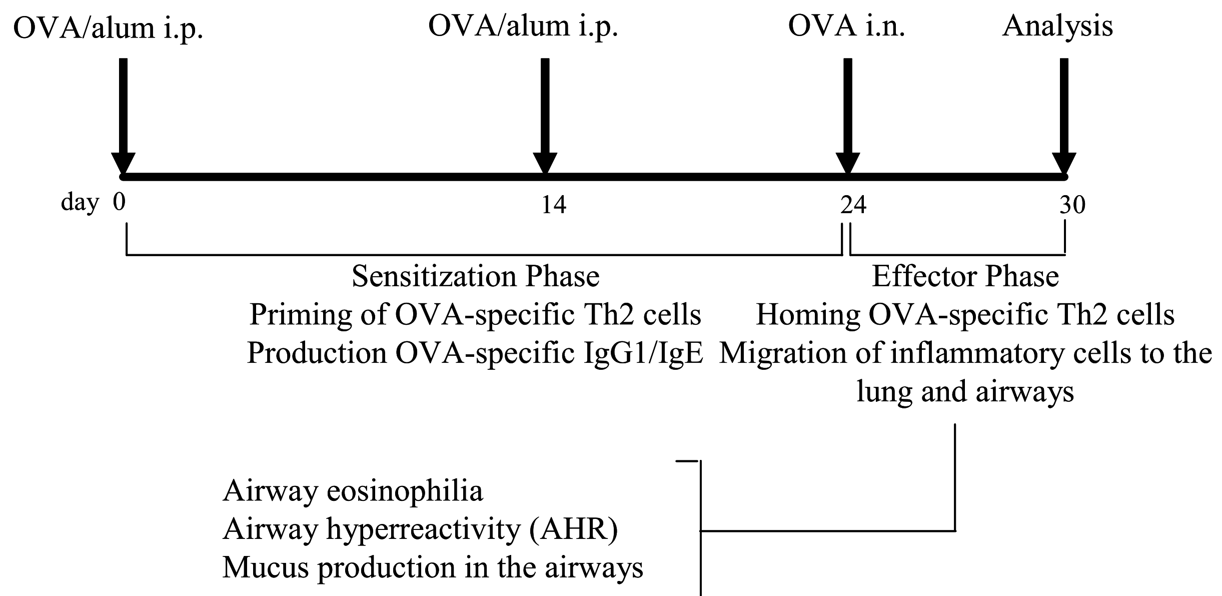

Figure 3. *Murine model to induce OVA-specific Th2 responses*

3.3. Bronchoalveolar lavage (BAL)

In order to evaluate allergic airway inflammation, mice were sacrificed, blood samples collected, the trachea cannulated and a BAL performed by flushing lung and airways four times with 1 ml PBS containing 10% fetal calf serum (FCS) (PAA laboratories, Linz, Austria). BAL cells were counted, spun onto glass slides using a cytospin (Shandon Southern Products Ltd., Asmoor, UK) and stained with Diff-Quick (Dade Behring, Marburg, Germany) according to the manufacturer’s instructions. Numbers of eosinophils, neutrophils, lymphocytes and macrophages present in the BAL were determined microscopically using standard histological criteria.

3.4. Th1-inducing adjuvants

BCG (Immunocyst Connaught, Ihringen, Germany) was heat-killed by incubating the mycobacteria twice for 1 h at 80°C in a water bath. The lack of viability of hk-BCG was monitored by plating serial dilutions onto plates containing Middlebrook 7H10 agar (Difco, USA) supplemented with 10% v/v Middlebrook ADC enrichment (Becton Dickinson, USA).

Colonies were counted after 21 days of incubation at 37°C in 9% CO_2. No colony forming units (CFUs) could be detected in the hk-BCG stock solution. Live BCG was used as positive control for viability and to verify the amounts of BCG being used for the immunizations. Phosphorothioate CpG oligodeoxynucleotides (CpG-ODN 1826, 5´-TCC ATG ACG TTC CTG ACG TT-3´) were synthesized by Sigma. PPD was purchased from Serum Statens Institute (Copenhagen, DK). No endotoxin was detected in any of the adjuvants using the Limulus assay (BioWhittaker, Inc., Walkersville, MD, USA). Live BCG or kh-BCG (2 x 10^6 CFU/mouse), CpG (30 μg/mouse) and PPD (40 μg/mouse) were applied to the mice together with OVA/alum during the OVA sensitization period as shown in figures 4, 8 and 13A.

3.5. Stimulation of bone marrow-derived dendritic cells (BM-DC)

BM-DC were generated from bone marrow of either BALB/c or C57BL/6 mice in the presence of high doses of GM-CSF (200 U/ml; PeproTech Inc, London, UK) as previously described (Lutz et al., 1999). Briefly, freshly prepared bone marrow cells were cultured in Click RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS (PAA laboratories, Linz, Austria), 2 mM L-glutamine, 10 mM HEPES buffer, 60 μg/ml penicillin, and 20 μg/ml gentamicin in the presence of 200 U/ml GM-CSF. Cultures were fed with additional 200 U/ml GM-CSF on days 3, 6 and 8. After 10 days, nonadherent cells were counted, resuspended at 1 x 10^6 cells/ml and pulsed on 24-well plates (NUNC, Denmark) for 18 h with either live BCG or hk-BCG (at 1 x 10^6 or 1 x 10^5 CFU/ml), CpG-ODN (30 μg/ml) or PPD (20 μg/ml). BM-DC resuspended only with supplemented RPMI medium were used as controls. The culture supernatants were harvested and tested for the presence of cytokines by ELISA. In parallel the expression levels of CD86, CD80 and MHC class II on the surface of the BM-DC were determined by FACS staining using the following mAbs (from Pharmingen, San Diego, CA) resuspended in FACS Buffer (PBS containing 2.5% FCS and 0.1% sodium azide (Merck Schuchardt, Hohenbrunn, Germany)):

FITC anti-mouse CD40 (HM40-3), PE anti-mouse CD86 (GL1), FITC anti-mouse CD80 (16-10A1) and PE anti-mouse I-Ab (AF6-120.1). Isotype-matched mAbs were used to control unspecific staining. Fluorescence intensity was measured using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Total RNA was also extracted 18 h after stimulation using the Rneasy Mini Kit from Qiagen (Hilden, Germany). To determine the mRNA abundance of the various chemokine receptors RNAse protection analyses (Kit purchased from Pharmingen) were performed following the manufacturer's instructions.

3.6. Treatment of mice with allergen-pulsed BM-DC

After washing, BM-DC pulsed with 25 μg/ml CpG (CpG-ODN 1668, 5'-TCCATGACGTTCCTGATGCT-3' obtained from Qiagen Operon, Cologne, Germany) and 100 μg/ml OVA (Sigma) were injected i.v. into the tail vein of naive mice on day -7 (5 x 10^5 per mice) (Figure 15A). Control mice were vaccinated with unpulsed BM-DC (maintained in the absence of either CpG or OVA). Subsequently, the protocol to induce OVA-specific Th2 responses was initiated.

3.7. Isolation and antigen pulsing of Langerhans cells (LC)

Epidermal LC suspensions were prepared from mouse ear skin by trypsinization procedures as previously described (Berberich et al., 2003). Briefly, the ventral thick ear halves were incubated in a solution of 1% trypsin in PBS for 90 min, and the dorsal thin ear halves were treated with 0.6% trypsin in PBS for 45 min. These preparations contained ~3-5% of LC that constitutively express MHC class II as well as MHC class II-negative keratinocytes, a source of GM-CSF that is essential for LC differentiation. The proportion of LC was determined by fluorescence labelling of MHC class II-positive cells. The preparations were completely devoid of macrophages, as documented by the lack of staining with the fluorescence-labelled mAb F4/80. For Ag pulsing, epidermal cell suspensions were cultured for 24 h in Click RPMI

1640 medium (Biochrome, Berlin, Germany), supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 10 mM HEPES buffer, 60 µg/ml penicillin and 20 µg/ml gentamicin, in the presence of 25 µg/ml OVA. Control cultures were set up in the absence of OVA (unpulsed LC). Thereafter, the cells were washed, resuspended in PBS and injected i.v. into the tail of naïve mice (3 x 10^5 cells/mouse). In these mice, the protocol to induce OVA-specific Th2 responses was initiated 7 days later.

3.8. *N. brasiliensis* parasites and preparation of NES

The *N. brasiliensis* lifecycle was maintained in Sprague-Dawley rats as previously described (Healer et al., 1991). Seven days after s.c. injection of 3000 infective stage (L3) larvae, animals were killed and adult worms were harvested from the gut by dissection. Worms were then washed repeatedly with sterile saline. Subsequently, they were preincubated in RPMI 1640 containing 1000 U/ml penicillin and 1 mg/ml streptomycin for 30 min at room temperature followed by five washes in RPMI 1640 before incubation in serum-free medium supplemented with 2% glucose, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C. Cultures contained approximately 1000 worms per ml medium. Medium was collected and replaced every 2-3 days during 14 days. NES was then prepared from parasite culture medium by 20-fold concentration using Millipore´s Amicon Ultra-15 centrifugal filter devices with a 5 kDa-retaining filter (Millipore Corporation) and filtered through a 0.2 µm filter (Schleicher & Schuell GmbH, Dassel, Germany). Protein concentration was determined by Bradford assay (Bio-Rad Laboratories GmbH, München, Germany). NES aliquots were stored at -20°C. Traces of endotoxin contamination (<1 µg/ml) were detected in all the NES stocks using the Limulus assay (BioWhittaker) however, LPS-depleted NES was prepared by using the Detoxi-Gel™ AffinityPak™ Pre-packed Columns (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Briefly, columns were regenerated by washing with five column volumes of 1% sodium deoxycholate (Sigma) followed by 6-10 column volumes of pyrogen-

free water to remove detergent and equilibrate the gel. NES was then applied to the column allowing the product to enter the gel bed. After incubating the columns for at least 1 h, LPS-depleted NES was collected by adding pyrogen-free buffer and then concentrated using the Amicon filter devices (Millipore). Protein concentration was again determined by Bradford assay. LPS contamination was then 100-fold reduced (<0.01 µg/ml). NES (50 µg/mouse) was applied to the mice together with OVA/alum during the OVA sensitization period as shown in figure 17A.

3.9. Digestion of NES with proteinase K

Proteinase K-agarose beads (Sigma) were suspended at 1 mg/ml in distilled water for 1 hour at 2-8°C to allow brief hydration and washed three times with ice-cold 20 mM Tris HCl at pH 7.2. The pellet was then mixed with 1 ml of the NES preparation (4000 µg protein/ml) at a 20% v/v ratio. The mixture was incubated at 50°C under agitation replacing the beads at 2, 24 and 48 hours. After 72 hours, the proteinase beads were removed by centrifugation for 1 min at 13000 rpm (in an Eppendorf microfuge) and the protein-digestion of the bead-free supernatant was then confirmed by Bradford assay (Bio-Rad Laboratories GmbH) and by silver staining of a polyacrylamide gel (Silver Stain Plus kit, Bio-Rad Laboratories GmbH). Prior to electrophoresis, the protein samples were mixed with loading buffer (4% SDS (sodium dodecylsulphate), 0.1 M Tris pH 8.9, 2 mM EDTA (ethylenediaminetetraacetic acid), 0.1% bromophenol blue, 20% glycerol, 0.2 M DTT (dithiothreitol)), heated at 95°C for 5 min and then cooled on ice. The 10-kDa protein ladder from GibcoBRL® (Invitrogen Corporation, Carlsbad, CA) was used as molecular weight marker. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on polyacrylamide slab gels (60 x 80 x 0.75 mm) using the discontinuous buffer system of Laemmli (Laemmli, 1970). The 4% stacking gel was overlaid on the separating gel of 12% polyacrylamide with an acrylamide:bis ratio 30:0.8. The running buffer consisted of 0.025 M Tris, 0.2 M Glycine and 0.1% SDS. After adding the samples, the

gels were run for approximately 2 h in a Mini-PROTEAN® 3 cell (Bio-Rad) at a constant current of 80V using the power supply model 200/2.0 from Bio-Rad. After SDS-PAGE, the gels were immediately fixed in a solution containing 50% methanol, 10% acetic acid and 10% fixative enhancer concentrate (Bio-Rad) for 20 min. Then, the gels were rinsed with desionized, destilled water and placed in the staining/developing solution containing silver nitrate (Bio-Rad). Silver reduction was allowed to proceed until a light yellow background appeared, ensuring maximum sensitivity. Image development was stopped by immersion of the gels in 5% acetic acid. Subsequently the gels were washed again with desionized, destilled water and dried.

3.10. Cell cultures

Single-cell suspensions from the mediastinal lymph nodes (MLN) were prepared by teasing the MLN through a steel mesh and discarding the cell debris. Total MLN cells were counted and resuspended at 2 x 10^6 cells/ml in RPMI medium (Sigma) supplemented with sodium bicarbonate (3.024 g/l), 10 µg/ml streptomycin, 10 U/ml penicillin, 50 µM 2-mercaptoethanol and 10% FCS. Cell preparations were added to cell culture plates (2 x 10^6 cells/ml) and left in medium alone, stimulated with 40 µg/ml OVA or with a plate-bound mAb to CD3ε (145-2C11, 25 µg/ml, Pharmingen) together with 200 U/ml recombinant human IL-2 (Novartis, Basel, CH) at 37°C in a humidified atmosphere containing 5% CO_2. After 48 h, the culture supernatants were harvested and tested for the presence of cytokines by ELISA.

3.11. Detection of immunoglobulins and cytokines by ELISA

OVA-specific Ig levels were determined by coating vinyl round bottom microtiter plates (Thermo Labsystems, Finland) overnight at 4°C either with OVA (10 µg/ml) or anti-IgE (R35-72, 1 µg/ml) and subsequently blocked with 10% FCS in PBS containing 0.05% Tween 20 (PBS-Tween) for 2 hours at room temperature (RT). After washing, 50 µl of serial serum

dilutions in PBS-Tween/1%FCS were applied overnight at 4°C. Antigen-antibody binding was detected using either biotinylated monoclonal antibodies against mouse IgG1 (A85-1) or IgG2a (R19-15) (4 μg/ml) or biotinylated OVA (for the detection of OVA-specific IgE) and visualized with a conjugate of biotinylated alkaline phosphatase/streptavidin (DAKO, Denmark) and p-nitrophenyl phosphate as substrate (Sigma). Absorbance was read at 405 nm with an ELISA microplate reader (Dynatech MRX). Serum titers are expressed as the reciprocal value of the serum dilutions, which were 2-fold over background optical density of serum from age-matched noninfected mice measured at 1/10 dilution (OD<0.1). Where indicated, NES-specific Ig ELISA were also performed. In this particular case, microtiter plates were coated overnight at 4°C with NES (10 μg/ml) and the NES-specific Ig titers were detected in the serum by using biotinylated monoclonal antibodies against mouse IgG1 (A85-1), IgG2a (R19-15) or IgE (R35-118).

For the detection of cytokines in the BAL fluid and cell culture supernatants, sandwich ELISA with the following mAbs, recognizing two different epitopes of the respective cytokines were used: unconjugated rat anti-mouse IL-5 (TRFK-5) and biotinylated rat anti-mouse IL-5 (TRFK-4), unconjugated rat anti-mouse IL-4 (BVD4-1D11) and biotinylated rat anti-mouse IL-4 (BVD6-24G2), unconjugated rat anti-mouse IL-13 (Clone 38213, R&D Systems, Minneapolis, MN) and biotinylated polyclonal goat anti-mouse IL-13 (R&D Systems), unconjugated rat anti-mouse IL-10 (JES5-2A5) and biotinylated rat anti-mouse IL-10 (SXC-1), unconjugated rat anti-mouse IFN-γ (R4-6A2) and biotinylated rat anti-mouse IFN-γ (XMG1.2). BAL fluid was concentrated 3-fold using Millipore´s Amicon Ultra-15 centrifugal filter devices with a 5 kDa-retaining filter (Millipore Corporation, Bedford, MA). When indicated, sandwich ELISA for the detection of TNF-α, IL-1β and IL-12p70 were also performed using the following mAbs: unconjugated rat anti-mouse TNF-α (MP6-XT22) and biotinylated rat anti-mouse TNF-α (MP6-XT3), unconjugated rat anti-mouse IL-1β (Clone

30311.11, R&D Systems) and biotinylated polyclonal goat anti-mouse IL-1β (R&D Systems), unconjugated rat anti-mouse IL-12p70 (9A5) and biotinylated rat anti-mouse IL-12p70 (C17.8). The further detection of the cytokines was performed as described before. For the quantification of the cytokines, titrations were performed using murine recombinant cytokines (Pharmingen). Unless otherwise indicated, all the antibodies were purchased from Pharmingen.

3.12. Staining of intracellular cytokines

$CD4^+$ T cells from the BAL producing IL-4, IL-5, IL-10 or IFN-γ were detected by using two and three-color FACS-analysis. For this purpose BAL cells from the different groups of mice were spun down and then resuspended in RPMI medium containing 10% FCS. The cells were then stimulated with phorbol ester (5 μg/ml) and calcium ionophore (0.5 μM) for 6 h (both reagents from Sigma) at 37°C in a humidified atmosphere containing 5% CO_2. Brefeldin A (2 μg/ml, Sigma) was added for the last 2 h of the *in vitro* culture period. The stainings were performed according to the instructions from Pharmingen. Briefly, after the 6 hours of stimulation, BAL cells were washed with FACS buffer (described in section 3.5) and stained with cychrome labeled anti-CD4 mAb (H129.19) for 15 min on ice in the dark. The cells were then fixed with 4% formalin in PBS for 20 min at RT in the dark and later incubated for additional 20 min with anti-CD16/CD32 mAb (2.4G2; Fc Block® (5 μg/ml)). After permeabilization with a buffer containing 1% FCS, 0.1% sodium azide and 0.1% saponin (Sigma) in PBS for 30 min at RT in the dark, the stained cells were either incubated with a mixture of PE-labeled anti-IL-4 (11B11)/FITC-labeled anti-IFN-γ (XMG1.2) or PE-labeled anti-IL-5 (TRFK-4)/FITC-labeled anti-IL-10 (JES5-16E3) mAbs resuspended in the permeabilization buffer at RT. Cells were then washed twice with permeabilization buffer and latter with FACS buffer and then analyzed using a FACSCalibur flow cytometer (Becton

Dickinson). Specificity of antibody binding was controlled by staining with isotype-matched control antibodies. All antibodies were purchased from Pharmingen.

3.13. Histological analysis

After performing the BAL, lungs were fixed in 10% phosphate-buffered formalin for 24 hours and embedded in paraffin wax. Sections (2-3 μm) were cut and stained using standard histological protocols with Haematoxylin and Eosin (H&E) (Sigma) or Periodic acid-Shiff reagent (PAS) (Sigma). The stained sections were visualized by light microscopy. The intensity of peribronchial and perivascular inflammation as well as the degree of goblet cell metaplasia was scored by two independent observers (0 = no inflammation or goblet cell metaplasia, 1 = slight inflammation or goblet cell metaplasia, 2 = strong inflammation or goblet cell metaplasia, 3 = very strong inflammation or goblet cell metaplasia).

3.14. Measurement of AHR

AHR was assessed by methacholine induced airflow obstruction, using a whole-body plethysmograph (model PLT UNR MS, EMKA TECHNOLOGIES, Paris) as previously described (Hamelmann et al., 1997). Pulmonary airflow obstruction was measured by Penh using the following formula: Penh = (Te/RT-1) x (PEF/PIF), where Penh = enhanced pause (dimensionless), Te = expiratory time, RT = relaxation time, PEF = peak expiratory flow (ml/s), and PIF = peak inspiratory flow (ml/s). Enhanced pause (Penh), minute volume, tidal volume and breathing frequency were obtained from chamber pressure, measured with a transducer connected to an amplifier module (model AC264) and analyzed by system XA software (version 1.565). Mice were exposed for 3 min to aerosolized 0.9% NaCl produced by a sonicator (model LS 290-990N), followed by incremental doses of aerosolized methacholine.

3.15. Cutaneous anaphylaxis

Active cutaneous anaphylaxis was tested six days after the OVA intranasal challenge in animals previously sensitized with OVA together with live or hk-BCG, CpG or PPD as described (Grunewald et al., 2002). Briefly, mice were injected i.v. with 200 µl 0.5% Evans blue in PBS. Subsequently the skin of the belly was shaved and 50 µl PBS containing 10, 1 or 0.1 µg/ml OVA or PBS alone was injected intradermally into four premarked sites on the skin. After 15 min, mice were killed and the skin was stripped off. Positive reactions towards allergen (OVA) resulted in IgE-mediated mast cell degranulation and fluid extravasation, which lead to the formation of a blue patch around the injection site. Two independent observers scored the degree of bluing as indicator for the intensity of mast cell degranulation (0 = no bluing, 1 = slight bluing, 2 = moderate bluing, 3 = strong bluing).

3.16. Adoptive transfer of cells

Mice were vaccinated as indicated in Figure 8A. Six days after the OVA i.n. challenge mice were sacrificed and single cell suspensions from spleens of each group of mice were prepared. In a separate experiment $CD4^+$ T cells were purified from the spleens of the different groups of mice using Cedarlane´s mouse CD4 cell recovery column kit following the manufacturer's instructions (Cedarlane Labs., Ontario, Canada). This procedure allowed the enrichment of mouse $CD4^+$ T cells leading to 85-95% purity as determined by FACS staining. Briefly, total spleen cells were incubated with a mixture containing mAbs directed against CD8 and the heat stable antigen (HSA, to deplete B cells) (both provided in the kit) for 30 min on ice. In parallel, the columns were washed with a cold balanced salt solution with 0.1% bovine serum albumin (BSS/BSA) adjusting the flow rate to 6-8 drops per minute by using a flow control valve. The cell suspensions were then applied to the column bed and the $CD4^+$ T cells were collected by repeated washing with BSS/BSA. 2 x 10^7 total spleen cells or 1 x 10^7 purified $CD4^+$ T cells (in 100 µl PBS) from the different groups of mice were i.v. injected into

untreated mice. One day later all the animals were subjected to the protocol that induces OVA-specific Th2 responses (see Fig. 3). A separate group of mice injected with no cells were also subjected to the Th2-inducing protocol as controls.

3.17. Generation of OVA-specific Th2 cells in vitro and application to mice

$CD4^+$ T cells were isolated from spleens of mice transgenic for the $OVA_{323-329}$-specific αβTCR (OT-2 mice) as described above. To obtain OVA-specific Th2 cells, OT-2-derived $CD4^+$ T cells were incubated *in vitro* in the presence of irradiated splenic DC pulsed with OVA peptide, IL-4 (1000 U/ml), IL-2 (human rIL-2; 1000 U/ml) and anti-IL-12 (C17.8, 10 μg/ml) and anti-IFN-γ (XMG1.2, 10 μg/ml) mAbs. Splenic DCs were isolated using metrizamide density-gradient according to the manufacturer's instructions (Cedarlane Labs.). After 5 days, the developed T cells were transferred to anti-CD3ε-coated 24-well culture dishes (145-2C11, 25 μg/ml, Pharmingen) and IL-2-containing culture medium (1000 U/ml) was added. After 5 additional days, the T cells were collected and transferred into C57BL/6 mice as indicated in Figure 12. Th2 cells produced large amounts of IL-4 and IL-5 (but very little IFN-γ) after *in vitro* restimulation with DC and OVA or antiCD3/IL-2 (data not shown). FACS analysis revealed that the T cell population obtained after reestimulation consisted of >99% $CD4^+V\alpha2^+$ T cells which identify the transgenic phenotype. Antibodies and cytokines were purchased from Pharmingen (mAb against Vα2 TCR, clone B20.1).

3.18. Treatment of mice with anti-IL-10R antibodies

Anti-IL-10R mAb were generously provided by Dr. M. Lutz (Erlangen, Germany). C57BL/6 mice were treated with anti-IL-10R mAb (320 μg/mouse i.p.) on days -1, 6, 13 and 20. As controls, groups of mice were treated with either PBS or isotype-matched mAb (also 320

μg/mouse i.p.). Simultaneously, the protocol to induce OVA-specific Th2 responses using either OVA/alum or OVA+NES/alum was initiated as described.

3.19. Statistical analysis

Results were evaluated using one-way ANOVA in order to establish differences between groups. Significant ANOVA were further analyzed by Dunns post hoc test. The student's *t*-test was also used when ANOVA was not appropriate.

4. **RESULTS**

4.1. *Efficacy of four different Th1-inducing adjuvants, i.e. live BCG, heat-killed BCG, CpG and PPD as components of vaccines aimed at inhibiting the development of allergic asthma*

4.1.1. Application of live BCG, hk-BCG, CpG-ODN or PPD together with OVA/alum reduces the development of allergen-induced airway inflammation

Previous studies have clearly shown that the application of allergen-specific Th1 cells or Th1-inducing adjuvants reduces the development of allergen-specific Th2 responses (Wohlleben and Erb, 2001) (Trujillo and Erb, 2003). In the present study, the capacity of four different Th1-inducing adjuvants to suppress allergen-specific Th2 responses *in vivo* was compared. For this purpose live or hk-BCG, CpG-ODN or PPD were applied together with OVA/alum i.p. (Fig. 4A) and subsequently, it was analyzed whether allergen-specific Th1 responses were initiated and allergen-specific Th2 responses (which normally develop after the application of OVA/alum) were suppressed. Figure 4B shows that the application of the different adjuvants together with OVA/alum resulted in a strong decrease in airway eosinophilia in comparison to the application of only OVA/alum in C57BL/6 mice. The strongest effect was observed using live BCG, which induced a 400-fold reduction in the number of eosinophils in the BAL. Hk-BCG, CpG-ODN and PPD also induced a significant but slighter reduction in airway eosinophilia (64, 6, and 17-fold reduction compared to OVA only treated mice, respectively). The decrease in eosinophils was accompanied by a significant reduction in the number of neutrophils, lymphocytes and macrophages when live BCG, hk-BCG or PPD but not CpG-ODN was used.

The reduction of airway eosinophilia also correlated with significantly reduced AHR (Fig. 4C) and decreased levels of IL-5 detected in the BAL fluid when the mice were treated with live BCG, hk-BCG or CpG-ODN (Fig. 4E). IFN-γ levels only increased in the airways of

mice vaccinated with live or hk-BCG. No IL-4 or IL-13 could be detected in the BAL fluid (data not shown). The application of live BCG, hk-BCG or CpG-ODN but not PPD also resulted in a significant decrease in the levels of IL-5 and IL-13 produced by MLN cells after *in vitro* stimulation with OVA (Fig. 4F). No significant differences in the levels of IL-10 or IFN-γ were found in the supernatants of the MLN cells from the different groups of mice stimulated with OVA (data not shown). Similar results were obtained, when the cells were restimulated with anti-CD3/IL-2 and no cytokines were detected when the cells were cultured in medium only. IL-4 could not be detected in any of the MLN cell cultures (data not shown).
Histological examinations of lung tissues from mice subjected to the OVA immunization protocol confirmed a strong reduction in the density of perivascular and peribronchiolar inflammation when live BCG was applied together with the allergen. Slighter but marked reduction in the inflammatory response was also observed when hk-BCG, CpG-ODN or PPD were used (data not shown). Figure 4D shows that mucus secretion and goblet cell metaplasia in the airways was virtually absent in mice treated with live and hk-BCG. The use of CpG-ODN and PPD also reduced mucus production and goblet cell metaplasia, but to a lower degree than live or hk-BCG (mean scores of the goblet cell metaplasia in the different groups: PBS: 0, OVA: 2, OVA+live BCG: 0, OVA+hk-BCG: 0, OVA+CpG: 0.5, OVA+PPD: 1).
Although all the tested compounds suppressed allergic inflammatory responses in the lung, only the application of live BCG and surprisingly PPD resulted in reduced OVA-specific IgG1 as well as OVA-specific IgE levels in the serum. Surprisingly, the application of hk-BCG induced a significant increase in the levels of OVA-specific IgE. On the other hand, application of live BCG, hk-BCG and CpG-ODN but not PPD led to increased levels of OVA-specific IgG2a in the serum (Fig. 4G). As the production of IgG2a is stimulated by IFN-γ, these results suggest that OVA-specific Th1 cells were generated *in vivo* after the application of live BCG, hk-BCG and CpG-ODN.

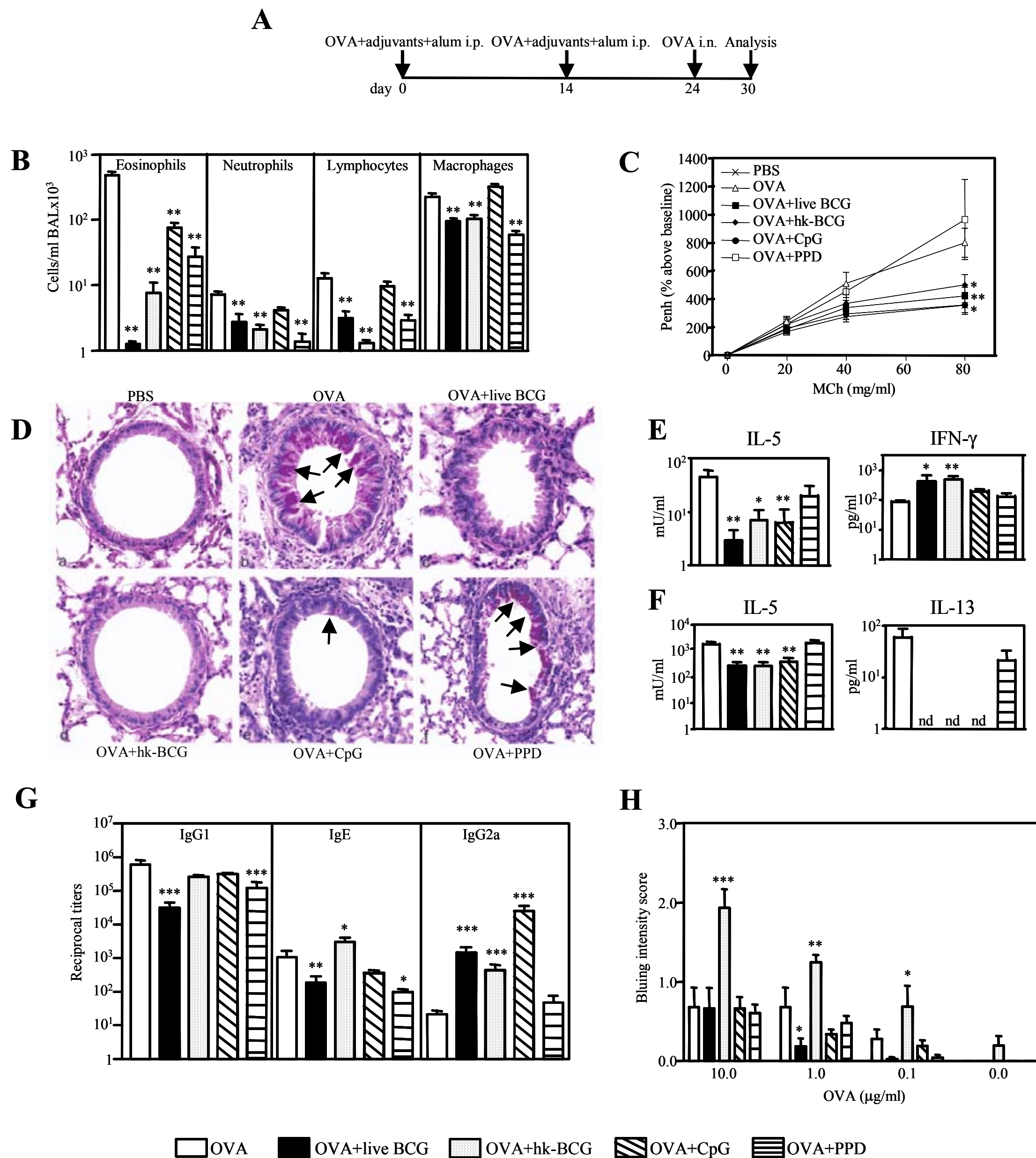

Figure 4. *Effect of the vaccination with live BCG, hk-BCG, CpG-ODN or PPD on OVA-specific allergic Th2 responses*. Mice were vaccinated with the different adjuvants during the OVA sensitization period (**A**). Shown are the absolute numbers of the different cell types present in the BAL (**B**), the AHR in response to increasing concentration of methacoline (six intranasal challenges were applied in this particular experiment on days 24, 25, 26 and 31, 32, 33 and AHR was measured on day 34) (**C**) and the mucus production in the airways (representative example of six mice/group are shown, 200x magnification) (**D**). Cytokine levels detected in BAL fluid (**E**) and in MLN cell cultures stimulated with OVA (**F**) are also depicted. At the bottom, the reciprocal titers of OVA-specific serum antibodies (**G**) and the intensity of bluing after performing an active cutaneous anaphylaxis test (**H**) are shown. Mean values of 7-12 mice/group ± SEM are shown. The experiments were repeated once (with the exception of C and H) with similar results. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to the values obtained from mice immunized only with OVA. Arrows indicate goblet cell metaplasia. n.d.: not detectable. The data shown in C were generously provided by Dr. Tobias Polte.

In order to confirm the results on the capacity of the different Th1-inducing adjuvants to modulate the production of allergen-specific IgE, the development of active cutaneous anaphylaxis was evaluated. This phenomenon is caused mainly by the immediate degranulation of mast cells in the skin after the crosslinking of FCεRI-bound allergen-specific IgE in their cell surface due to the intradermal application of the allergen. In agreement with the capacity of live BCG to decrease the serum levels of OVA-specific IgE (see Fig. 4G), its application resulted also in significantly reduced cutaneous mast cell reactivity after the intradermal application of OVA in comparison to that in OVA/alum only injected mice (Fig. 4H). Remarkably, the vaccination with hk-BCG together with OVA/alum induced an substantial increase in the cutaneous mast cell reactivity after intradermal application of OVA (Fig. 4H). This finding correlated with the capacity of hk-BCG to increase OVA-specific IgE serum antibodies (see Fig. 4G). On the other hand, the application of CpG or PPD was not able to decrease the cutaneous mast cell reactivity.

In summary, when the different Th1-inducing adjuvants were applied together with OVA/alum during the OVA sensitization protocol, live BCG>>hk-BCG>CpG>PPD were able to downmodulate the allergic Th2 responses and induce protective allergen-specific Th1 cells. No evidence of increasing inflammation in the lung or BAL was found by the i.p. application of the different adjuvants when the protocol showed in Figure 4A was used.

4.1.2. Adjuvant-induced suppression of allergic Th2 responses is dependent on IL-12 and IFN-γ

The reduction of allergen-induced Th2 responses by the application of live BCG, hk-BCG or CpG-ODN was associated with increased Th1 responses. This suggests that Th1 cells secreting IFN-γ were responsible for this effect. However, recently it was reported that mycobacterial induced suppression of Th2 responses may also be due to Tr and not Th1

responses (Zuany-Amorim et al., 2002). Furthermore, Kline et al. showed that the application of CpG-ODN could still inhibit the development of allergen-specific Th2 responses in the absence of IFN-γ or IL-12 (Kline et al., 1999). To confirm that the observed adjuvant-induced suppression of allergic-Th2 responses were due to the generation of Th1 responses, the experiments described in Figure 4A were repeated in IFN-γ- and IL-12 p35-deficient mice.

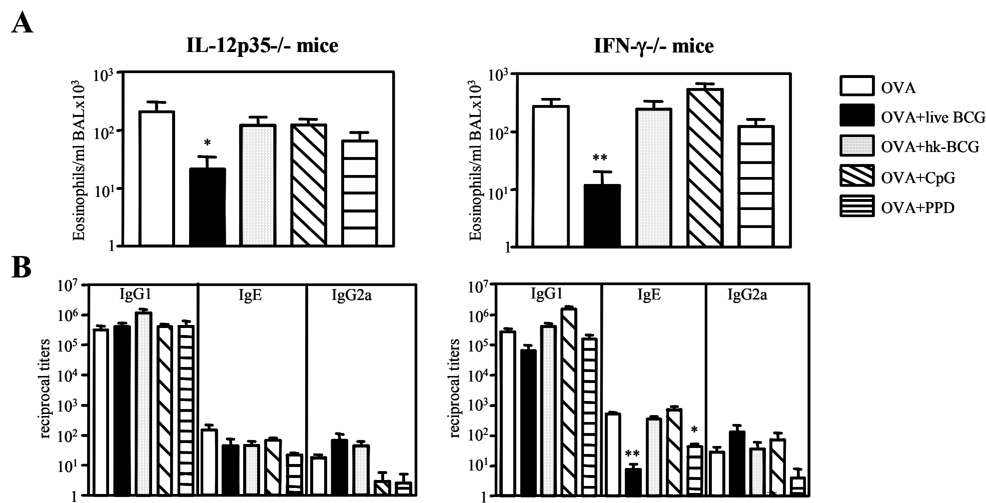

Figure 5. *Anti-allergic effect of the different Th1-inducing adjuvants in the absence of either IL-12p35 or IFN-γ.* IL-12p35- or IFN-γ-deficient mice were vaccinated i.p. with live BCG, hk-BCG, CpG-ODN or PPD as described in Figure 4A. Shown are the absolute numbers of eosinophils present in the BAL (**A**) and the reciprocal titers of OVA-specific serum antibodies (**B**) from the different groups (7-8 mice/group) in either IL-12p35- or IFN-γ-deficient mice. *p<0.05 and **p<0.01 compared to the values obtained from mice immunized only with OVA.

Figure 5 shows that the suppressive effect of hk-BCG, CpG-ODN or PPD on allergen-induced eosinophilia was totally dependent upon IL-12 and IFN-γ. In contrast, in the presence of live BCG, eosinophil numbers were still significantly reduced indicating that this effect was only partially dependent on either one of these two cytokines. The reduction of allergen-specific IgE and IgG1 levels observed in mice treated with live BCG or PPD was dependent upon IL-12 but could still be observed in IFN-γ-deficient mice. In contrast, the increase in OVA-specific IgG2a serum levels induced in mice treated with live BCG, hk-BCG or CpG-ODN was not observed in either IL-12- or IFN-γ-deficient mice.

To assess whether live BCG mediated the suppressive effects on Th2 responses by generating IL-10-producing Tr cells or alternatively by inducing IL-23 (a heterodimer composed of the IL-12p40 subunit and the IL-12p35-related molecule p19 which acts also on Th1 effector/memory $CD4^+$ T cells), the experiments were repeated in IL-10- and IL-12p35/p40-deficient mice. Interestingly, live BCG-mediated suppressive effect on allergen-induced airway eosinophilia was still observed in both IL-10- and IL-12p35/p40-deficient mice (Fig. 6).

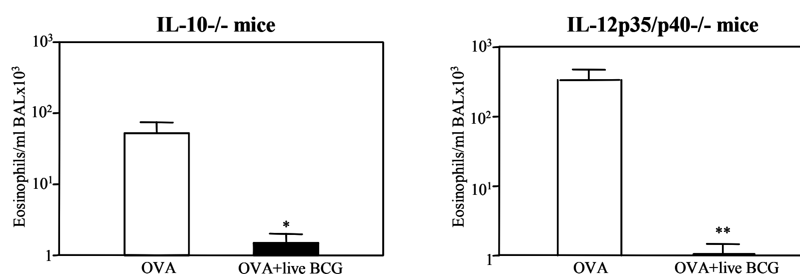

Figure 6. *Suppressive effect of live BCG on allergen-induced airway eosinophilia in either IL-10- or IL-12p35/p40-deficient mice.* IL-10- or IL-12p35/p40-deficient mice were vaccinated i.p. with OVA+live BCG as described in Figure 4A. Shown are the mean ± SEM of the absolute numbers of eosinophils present in the BAL of vaccinated mice (7-8 mice/group) in comparison to those in mice immunized only with OVA. *$p<0.05$ and **$p<0.01$.

4.1.3. TLR-2, TLR-4 and MyD88 are not necessary for the anti-allergy effects of live BCG

To further identify which factors were involved in the suppressive effect of live BCG on allergen-induced airway eosinophilia, the protocol shown in Fig. 4A using live BCG was applied to TLR-2/TLR-4-double deficient mice or MyD88-deficient mice. BCG contains molecules which are known to interact with TLR-2 and TLR-4 on the surface of APC (Seya et al., 2002) initiating a signaling cascade leading to nuclear factor kappa B (NF-κB) activation through transduction pathways that partly involve the adaptor protein MyD88. NF-κB activation mediates the production of proinflammatory cytokines and the expression of costimulatory molecules by APC contributing to the generation of specific T-cell responses

(Takeda and Akira, 2005). Therefore, it is conceivable that a TLR-mediated mechanism could be implicated in the capacity of live BCG to downmodulate allergen-specific T cell responses. Surprisingly, neither the absence of TLR-2/TLR-4 nor the absence of MyD88 could abolish the ability of live-BCG to suppress the OVA-induced hypereosinophilia in the airways (Fig. 7).

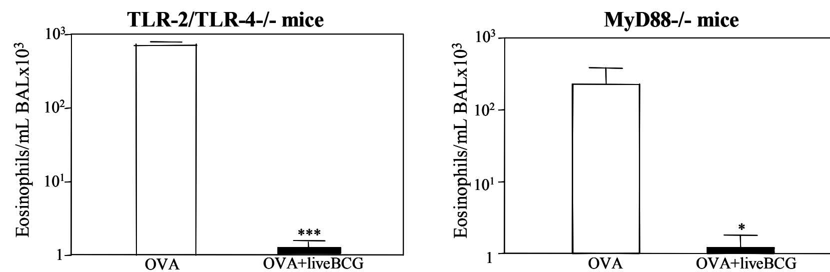

Figure 7. *Suppressive effect of live BCG on allergen-induced airway eosinophilia in either TLR-2/TLR-4- or MyD88-deficient mice.* TLR-2/TLR-4- or MyD88-deficient mice were vaccinated i.p. with OVA+live BCG as described in Figure 4A. Shown are the mean ± SEM of the absolute numbers of eosinophils present in the BAL of vaccinated mice (6-7 mice/group) in comparison to those in mice immunized only with OVA. *p<0.05 and ***p<0.001.

4.1.4. Application of live BCG and hk-BCG but not CpG-ODN or PPD reduces allergen-induced airway inflammation after secondary sensitization with the allergen

The results described in the previous sections suggest that inducing allergen-specific Th1 responses by the application of the different adjuvants may be useful for the development of an anti-asthma vaccine in humans. However, an efficient vaccine should not only protect from Th2 cell development shortly after its application but most importantly when individuals are re-exposed to the allergen. To evaluate whether the tested vaccine candidates fulfil these criteria, the inhibitory effect of live or hk-BCG, CpG-ODN or PPD applied together with OVA/alum on the development of allergic Th2 responses was examined after a second period of OVA/alum sensitization (Fig. 8A).

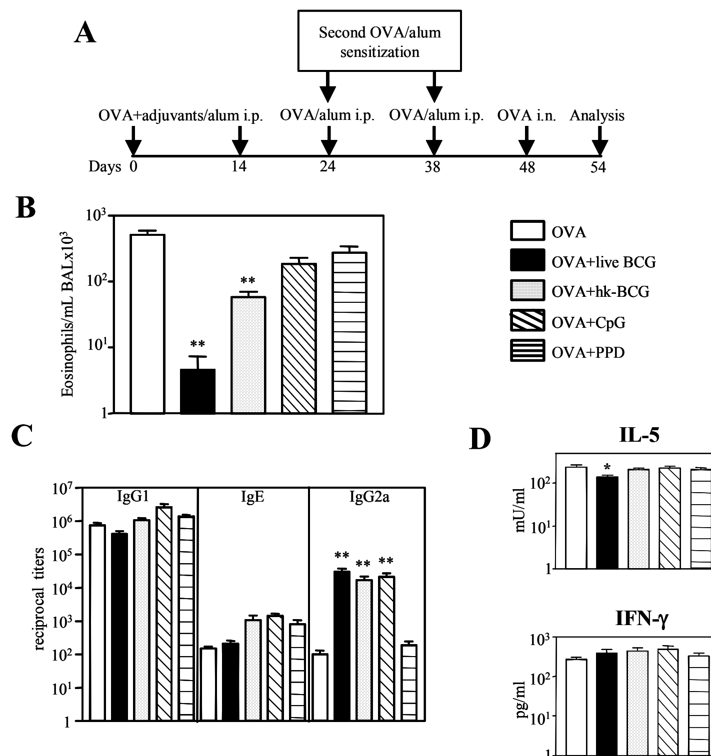

Figure 8. *Effect of vaccination with live BCG, hk-BCG, CpG-ODN or PPD on OVA-specific allergic Th2 responses in mice subjected to a second OVA/alum sensitization.* The ability of the different Th1-inducing adjuvants applied together with OVA/alum i.p. to inhibit OVA-specific allergic Th2 responses was also analyzed after a second period of OVA/alum sensitization (**A**). Shown are the mean ± SEM of the absolute numbers of eosinophils in the BAL (**B**), the reciprocal titers of OVA-specific serum antibodies (**C**) and the cytokine levels in the BAL fluid (**D**) of 18-23 mice/group from three separate experiments. *p<0.05 and **p<0.01 compared to the values obtained from mice immunized only with OVA.

Figure 8B shows that using this protocol the application of live BCG was able to induce a 320-fold reduction in the generation of OVA-induced eosinophilia in the airways. The application of hk-BCG also significantly reduced airway eosinophilia but to a lesser extent compared to its effect using the protocol shown in Figure 4A (7-fold reduction in comparison to 64-fold reduction using the protocol shown in Fig. 4A). Most importantly, the initial suppressive effect of CpG-ODN or PPD on Th2 responses in the lung were no longer present after a second sensitization with OVA. No changes in the levels of allergen-specific IgG1 and IgE serum levels were found with any of the adjuvants used (Fig. 8C). Similar to the results

shown in Figure 4E, it was again found that the application of live BCG, hk-BCG or CpG-ODN resulted in increased levels of OVA-specific IgG2a in the serum (Fig. 8C). Only the reduction in the airway eosinophilia induced by live BCG was associated with decreased levels of IL-5 in the BAL (Fig. 8D). Although increased levels of IFN-γ in the BAL of mice treated with the different adjuvants were found, the amounts were not significantly higher than in the OVA only treated group (Fig. 8D). Also no significant changes in the levels of IL-5, IL-13, IL-10 or IFN-γ in OVA-stimulated MLN cell cultures from the different groups of mice could be observed, and no IL-4 was detected in the BAL fluid or in any of the culture supernatants (data not shown).

The examination of lung sections revealed that the application of live BCG or hk-BCG strongly reduced pulmonary inflammation, goblet cell metaplasia and mucus production in mice sensitized with OVA. In contrast, the application of CpG-ODN or PPD only led to a slight reduction in OVA-induced airway inflammation (Fig. 9). Interestingly, mucus production and goblet cell metaplasia were reduced in CpG-ODN but not PPD treated mice in comparison to OVA only sensitized animals (Fig. 9) (mean scores of pulmonary inflammation/goblet cell metaplasia in the different groups: PBS: 0/0, OVA: 3/3, OVA+live BCG: 1/0, OVA+hk-BCG: 1/0, OVA+CpG: 2/1, OVA+PPD: 2/3).

These effects correlated with decreased percentages and absolute numbers of $CD4^{+}IL\text{-}4^{+}$ and $CD4^{+}IL\text{-}5^{+}$ cells detected in the BAL (Fig. 10). No significant difference in the percentage and absolute numbers of $CD4^{+}IL\text{-}10^{+}$ cells was observed (Fig. 10). In addition, the vaccination with live BCG, hk-BCG or CpG-ODN together with OVA/alum induced an increase in the percentage of $CD4^{+}IFN\text{-}\gamma^{+}$ cells detected in the BAL after secondary sensitization and challenge with OVA although the absolute numbers of IFN-γ-producing CD4+ cells remained unaltered (Fig. 10).

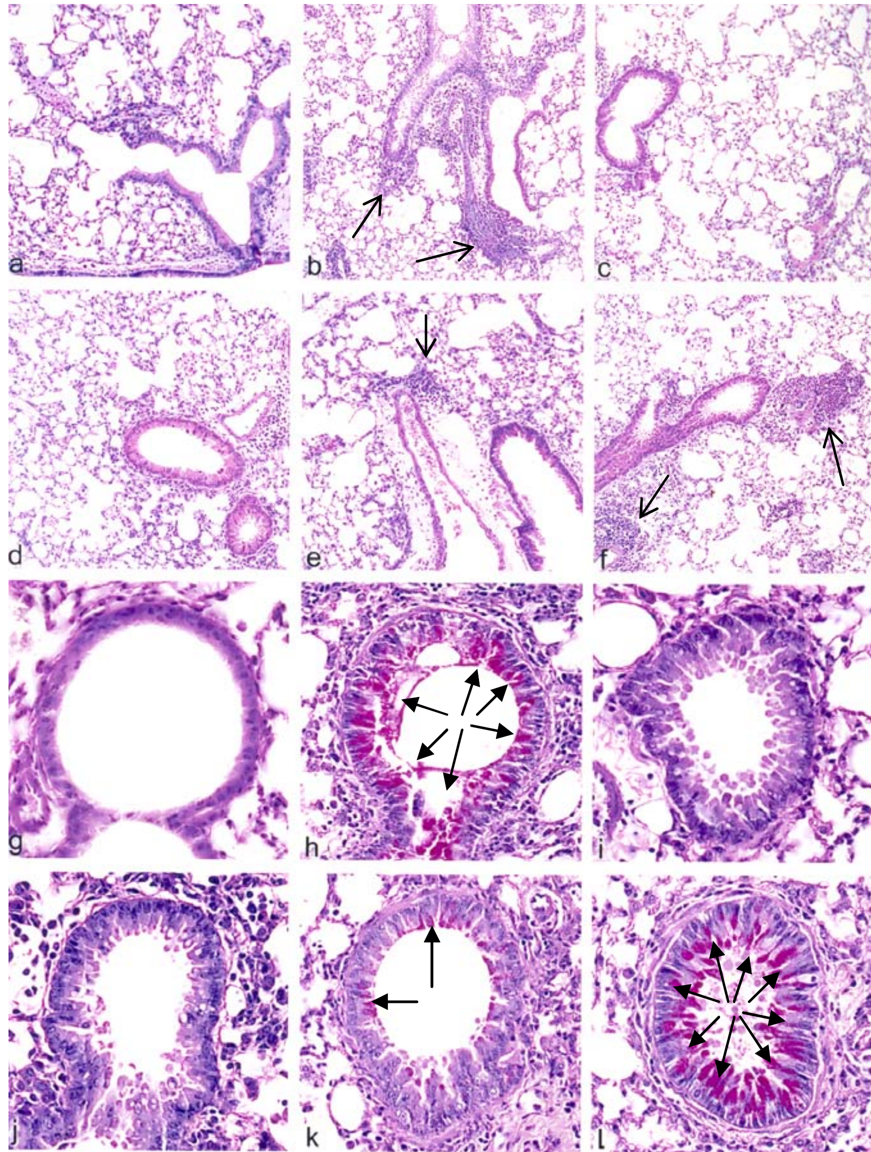

Figure 9. *Effect of the different Th1-inducing adjuvants on the development of airway inflammation and goblet cell metaplasia after a second OVA/alum sensitization.* Mice were vaccinated as described in Figure 8A. Shown are lung sections subjected to H&E (a-f) or PAS (g-l) staining from PBS-treated mice (a,g), OVA-immunized mice (b,h), live BCG (c, i), hk-BCG (d, j), CpG-ODN (e,k) or PPD-vaccinated mice (f,l). Representative examples of six mice/group are shown (a-f, 20x and g-l, 200x magnification). Open arrows indicate areas with peribronchial or perivascular inflammation, closed arrows indicate goblet cell metaplasia.

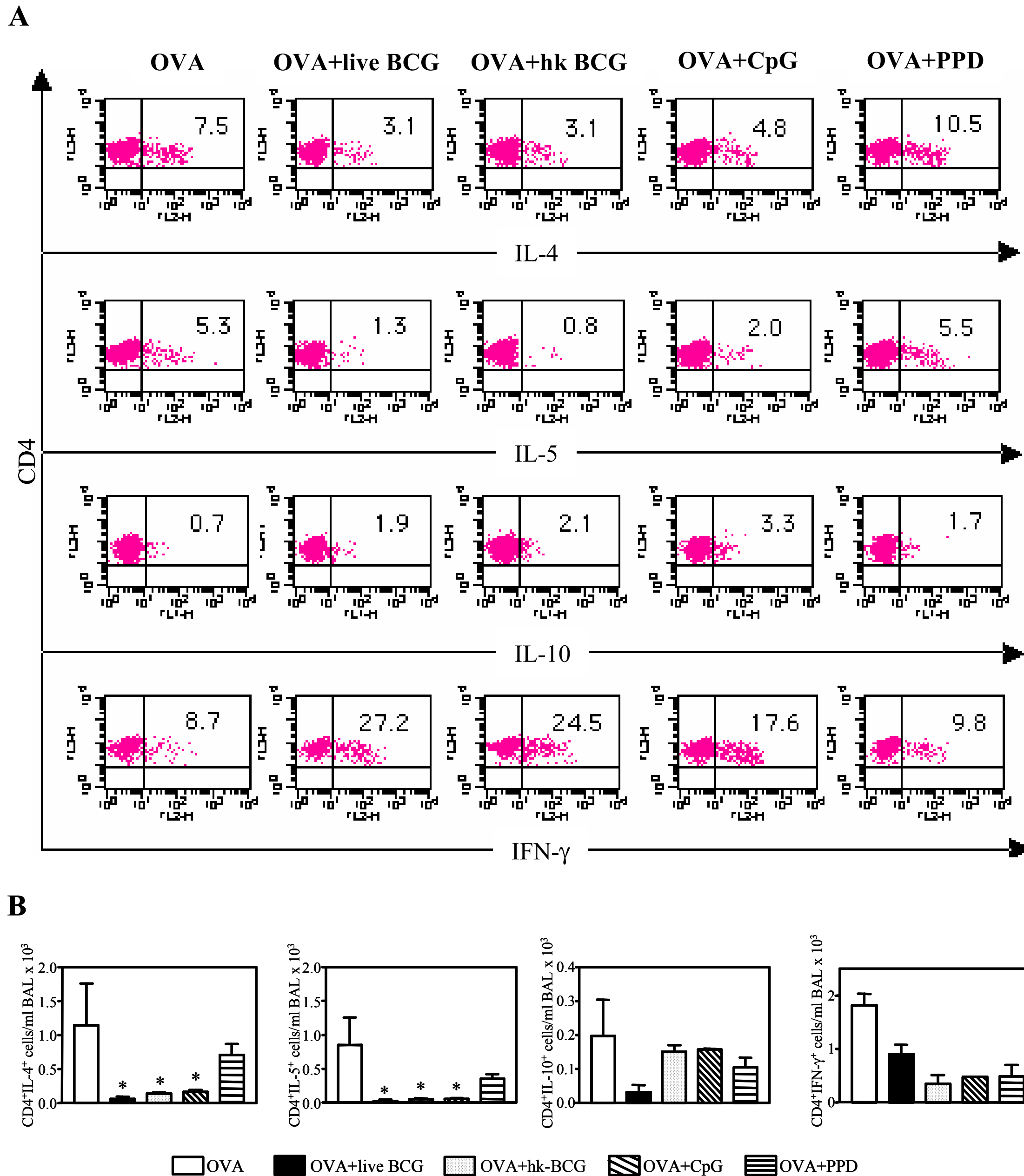

Figure 10. *Effect of the different Th1-inducing adjuvants in the percentage and absolute numbers of IL-4-, IL-5-, IL-10- and IFN-γ-secreting cells present in the BAL after a second OVA/alum sensitization.* Mice were vaccinated as described in the Figure 8A. BAL cells were then counted, stimulated, fixed and stained for the intracellular expression of cytokines as described in *Materials and Methods*. Shown are FACS dot plots indicating the percentages of $CD4^+$ T cells producing IL-4, IL-5, IL-10 or IFN-γ (representative of six mice/group) (**A**). The absolute numbers of $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}5^+$, $CD4^+IL\text{-}10^+$ and $CD4^+IFN\text{-}\gamma^+$ cells in the BAL is also shown (**B**).

Additionally, the duration of the protective effect of live and hk-BCG on the development of OVA-induced airway inflammation was analyzed. When mice were sensitized for a second time with OVA/alum starting three months after the last i.p. application of live BCG or hk-BCG together with OVA/alum, they still showed a significant reduction in airway eosinophilia in comparison to OVA only immunized mice (Table I).

TABLE I. *Absolute numbers of the different cell types present in the airways of mice vaccinated with live BCG, hk-BCG, CpG-ODN or PPD three months before a second OVA/alum sensization.*

		OVA	OVA+live BCG	OVA+hk-BCG	OVA+CpG	OVA+PPD
Cells/ml BALx10^3	Eosinophils	497.6 ± 131.8	44.1 ± 13.7*	101.0 ± 26.0*	465.8 ± 205.9	240.0 ± 97.5
	Neutrophils	8.7 ± 1.8	3.0 ± 0.5	6.5 ± 1.7	9.6 ± 2.9	3.6 ± 1.4
	Lymphocytes	8.8 ± 0.9	3.6 ± 0.5	6.0 ± 1.8	16.0 ± 5.5	4.1 ± 1.3
	Macrophages	321.5 ± 43.9	226.5 ± 48.3	333.8 ± 36.1	385.7 ± 201.9	177.4 ± 52.4

Mice were vaccinated as described in Figure 8A with the exception that the timepoint between the last i.p. application of the adjuvants and the beginning of the second round of OVA/alum sensization was 12 instead of 2 weeks. The absolute numbers of eosinophils, neutrophils, lymphocytes or macrophages in the BAL of mice are shown (mean numbers of 5-6 individual mice/group ± SEM). The experiment was repeated once with similar results. *p<0.05 compared to the values obtained from mice immunized only with OVA.

Mice were also vaccinated subcutaneously instead of intraperitoneally with the different adjuvants together with OVA/alum (Fig. 8A). By using subcutaneous vaccination, live BCG was the only adjuvant able to significantly reduce airway eosinophilia (OVA: 501.6±72.4; OVA+live BCG: 208.0±61.4*; OVA+hk-BCG: 280.7±41.4; OVA+CpG: 654.4±143.8; OVA+PPD: 449.0±77.2 eosinophils/ml BALx10^3 (mean of 9-15 mice/group from two separate experiments ± SEM; *p<0.05 between OVA and OVA+live BCG groups).

4.1.5. Adoptive transfer of total spleen cells or splenic $CD4^+$ T cells from mice immunized with the allergen and live or hk-BCG reduces allergen-induced pulmonary eosinophilia

Next it was investigated whether the suppressive effects induced by the different adjuvants on the development of allergic Th2 responses were mediated by lymphocytes. For this purpose, single cell suspensions were prepared from spleens of mice, which had been vaccinated with the different adjuvants or OVA/alum alone and subjected to a second round of OVA/alum sensitization (Fig. 8A). The isolated spleen cells were transferred i.v. into naive mice as described in *Materials and Methods*. One day after cell transfer the mice were subjected to the OVA immunization protocol (Fig. 3). As controls, naïve mice not transferred with any cells were also included. Figure 11 shows that the adoptive transfer of spleen cells from mice injected either with live or hk-BCG but not CpG-ODN or PPD (together with OVA/alum) resulted in a significant reduction in the numbers of eosinophils in the BAL compared to the control mice transferred with spleen cells from OVA/alum only treated mice or mice transferred with no cells. OVA-specific IgG1 and IgE levels in the serum were not affected by the adoptive transfer of spleen cells from any of the different groups of mice. In contrast, the transfer of spleen cells from live BCG, hk-BCG or CpG-ODN but not PPD immunized mice resulted in strongly enhanced OVA-specific IgG2a serum levels.

The experiment was repeated using purified $CD4^+$ T cells from the spleen of live BCG and hk-BCG treated mice. Figure 11 shows that the results were similar to those found when using total spleen cells, suggesting that the application of live and hk-BCG resulted in the generation of $CD4^+$ T cells which protected mice from the development of airway eosinophilia but did not inhibit the production of allergen-specific IgE. The greatly enhanced OVA-specific IgG2a levels detected in the protected mice indicate that the transferred cells contained allergen-specific Th1 cells.

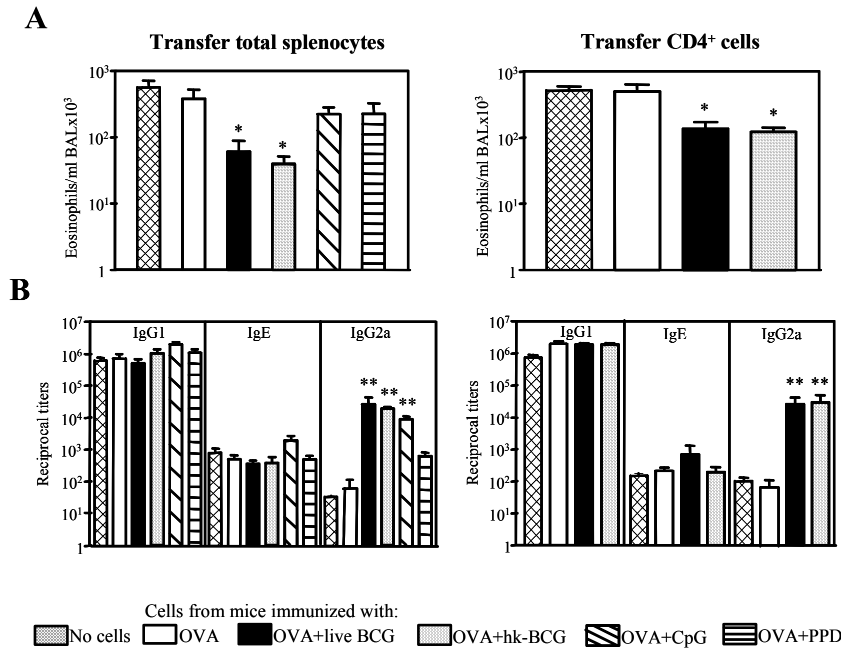

Figure 11. *Adoptive transfer of spleen cells from mice vaccinated with the different Th1-inducing adjuvants.* Suspensions of total splenocytes or purified CD4+ T cells were prepared from mice vaccinated as described in Figure 8A. Naïve C57BL/6 mice were then injected with these cells and one day later subjected to the protocol which induces OVA-specific Th2 responses as described in Figure 3. Shown are the absolute numbers of eosinophils in the BAL (**A**) and the serum titers of OVA-specific antibodies (**B**) in the different groups of mice (mean of 7-9 mice/group ± SEM). *p<0.05 and **p<0.01 compared to the values obtained in mice adoptively transferred with cells from mice immunized only with OVA.

4.1.6. Application of live BCG reduces the number of Th2 cells present in the airways after allergen airway challenge

In the previous experiments, live BCG was demonstrated to be the strongest Th2 response inhibitor in comparison to hk-BCG, CpG and PPD. Live BCG was able to suppress the development of airway eosinophilia and the amount of IL-5 present in the BAL fluid even after secondary OVA sensitization. Moreover, these effects seemed to be mediated by $CD4^+$ T cells. Based on recent reports suggesting that Th2 cells show impaired homing into Th1 cell-mediated inflamed sites (Wohlleben et al., 2003), it is possible that live BCG reduced the pulmonary allergic response by interfering with the recruitment of Th2 cells into the airways. In order to confirm this hypothesis, OVA-specific $CD4^+$ Th2 cells were generated *in vitro*

(derived from OT-2 transgenic mice on a C57BL/6 background) and injected i.v. into control mice or mice that have been previously subjected to the i.p. application of either OVA/alum or OVA+live BCG/alum as described in Figure 12A. The mice were then treated twice with OVA i.n. to induce the recruitment of the injected Th2 cells into the airways (Fig. 12A).

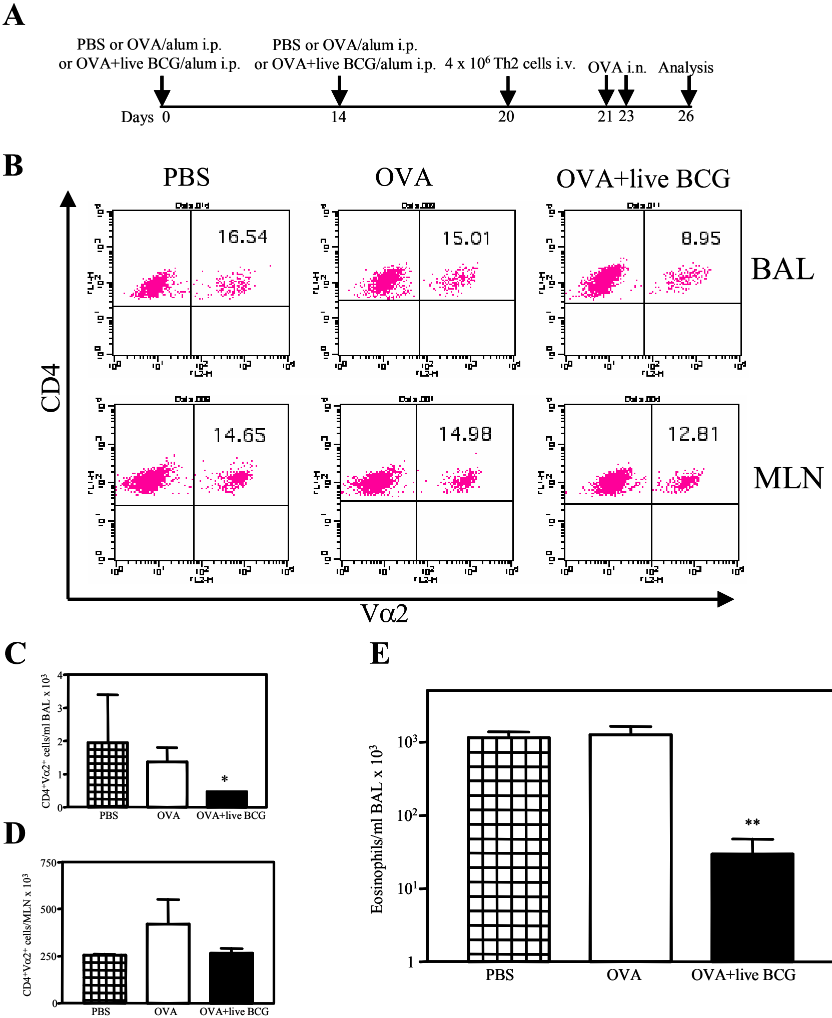

Figure 12. *Effect of live BCG on the recruitment of in vitro-generated OVA-specific $CD4^+$ Th2 cells into the airways.* OVA-specific Th2 cells derived from OT-2 TCR transgenic mice were generated as described in *Materials and Methods* and then injected i.v. into control mice (receiving only PBS) or mice that have been previously sensitized with either OVA/alum or OVA+live BCG/alum (**A**). The percentages of $CD4^+$ T cells expressing the transgenic TCR ($V\alpha2$) in pooled cells from BAL or MLN are indicated (**B**). Total amounts of $CD4^+V\alpha2^+$ cells present in the BAL and MLN (**C and D**) and the absolute numbers of eosinophils in the BAL (**E**) are also shown (mean of 7-9 mice/group ± SEM). *p<0.05 and **p<0.01 compared to the values obtained from mice immunized only with OVA.

Figures 12B, C and D, show that the percentage and the total numbers of $CD4^+$ T cells expressing the transgenic TCR were significantly reduced in BAL (but not in the MLN) after application of OVA+live BCG/alum in comparison to the application of PBS or OVA/alum. Furthermore, airway eosinophilia induced by the transferred Th2 cells was also strongly reduced after the vaccination with live BCG (Fig. 12E). The i.n. application of OVA without either OVA/alum sensitization or transfer of Th2 cells did not lead to an increase in eosinophils in the airways (data not shown).

4.1.7. Immunization with live BCG together with the allergen results in increased inflammatory responses but not AHR or goblet cell metaplasia in the airways after repeated allergen challenges

None of the experiments showed before have provided evidence that Th1 mediated inflammation occurred in the lungs of the mice vaccinated with the different Th1-inducing adjuvants. However, their effects were so far only investigated after a single i.n. application of OVA. It could be possible that after repetitive exposures to the allergen, allergen-specific Th1 cells may increase in numbers and lead to inflammation and tissue destruction in the airways. To test this hypothesis mice were immunized with OVA+live BCG/alum (the strongest Th2 response inhibitor) and then, they were subjected to 9 repetitive OVA airway challenges (Fig. 13A). Figure 13B shows that indeed the number of macrophages and neutrophils increased in the BAL of these mice. However, mice vaccinated with OVA+live BCG/alum still exhibited significantly reduced numbers of eosinophils in the airways and did not develop AHR or goblet cell metaplasia in comparison to mice sensitized only with OVA/alum (Fig. 13B, C and D) (mean scores of goblet cell metaplasia: PBS: 0, OVA/alum: 3, OVA+live BCG/alum: 0). Moreover, perivascular and peribronchial inflammation were still decreased when OVA+live BCG/alum was applied before repetitive i.n. challenges (mean scores of pulmonary

inflammation: PBS: 0, OVA/alum: 3, OVA+live BCG/alum: 2). BCG needed to be alive to inhibit the development of AHR, since using hk-BCG instead of live BCG did not result in the suppression of AHR (data not shown).

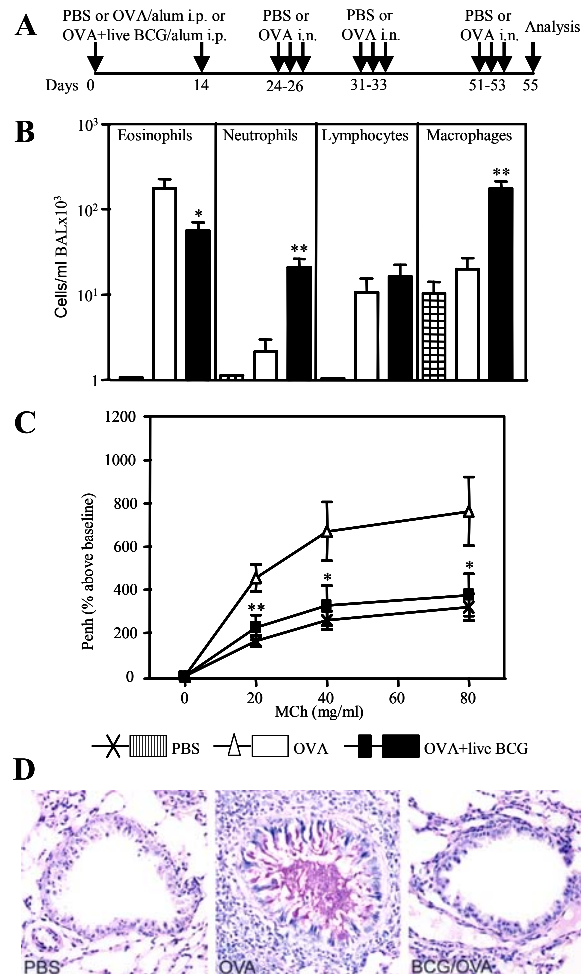

Figure 13. *Effect of repeated OVA i.n. challenges after vaccination with OVA+live BCG/alum.* Mice were vaccinated with OVA+live BCG/alum and then subjected to repeated i.n. challenges (**A**). Shown are the absolute numbers of the different cell types in the BAL (**B**) and the AHR in response to increasing concentration of methacoline (**C**) (mean ± SEM from 8-10 mice/group). *p<0.05 and **p<0.01 compared to the values obtained from mice immunized only with OVA. Goblet cell metaplasia and mucus production in the airways were also evaluated (**D**). Shown are representative examples of the lung sections from 6-8 mice/group (200x magnification). The data shown were generously provided by Dr. Tobias Polte.

4.1.8. Effect of the different adjuvants on the maturation and cytokine production of BM-DC *in vitro*

Finally, it was investigated whether live BCG was also more effective than the other adjuvants in inducing the maturation of BM-DC. Figure 14A shows that live BCG and hk-BCG were more effective than CpG-ODN or PPD in increasing the surface expression of CD80 and CD86 on BM-DC generated *in vitro*. Figure 14B shows that all the adjuvants except PPD induced the downregulation of the chemokine receptors CCR5 and CCR2, another indicative of BM-DC maturation. However, live BCG was the only stimulus able to induce simultaneously the secretion of high levels of TNF-α, IL-1β and IL-12p70 by these cells (Figure 14C). Live and hk-BCG but not CpG-ODN or PPD were also able to induce the production of IL-10 by BM-DC when used at high concentrations. As expected, CpG-ODN induced the secretion of large amounts of bioactive IL-12p70.

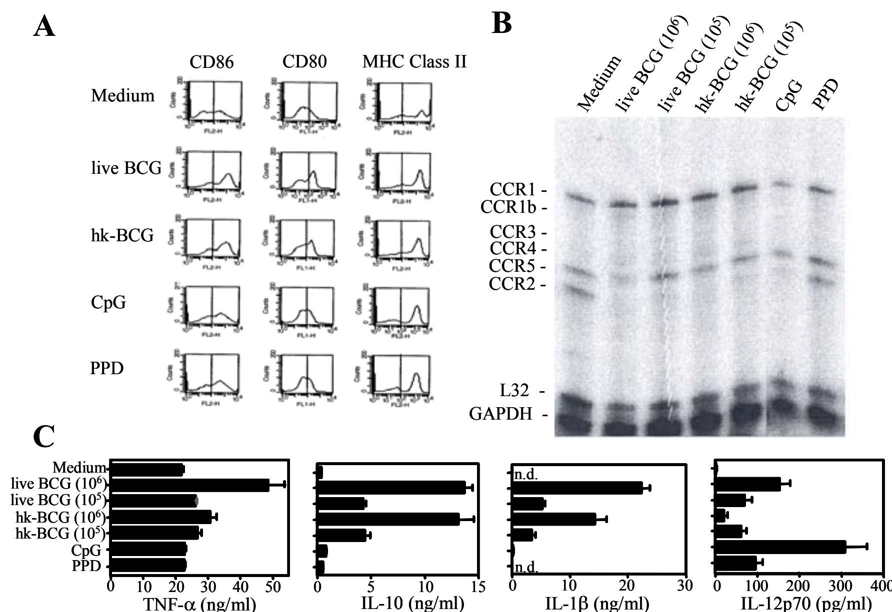

Figure 14. *Activation of BM-DC by live BCG, hk-BCG, CpG-ODN or PPD.* BM-DC were prepared and stimulated with the different adjuvants as described in *Materials and Methods*. Shown are the surface expression levels of CD86, CD80 and MHC class II (**A**), the RNA transcripts for the different chemokine receptors (**B**) and the cytokine levels in the supernatants of BM-DC cultures after 18 h stimulation (**C**). The results are representative for two separated experiments. n.d.: not detectable. The data shown in B were generously provided by Dr. Alois Palmetshofer.

4.2. *Assessment of a dendritic cell-based vaccination strategy to suppress the development of allergen-specific Th2 responses*

4.2.1. Application of BM-DC pulsed with CpG/OVA does not lead to the suppression of OVA-induced Th2 responses in BALB/c mice

The main goal of this part of the thesis was to design a DC-based vaccination strategy leading to the induction of allergen-specific Th1 cells, which then in turn protect mice from developing allergen-specific Th2 responses. Since BM-DC activated by CpG DNA *ex vivo* in the presence of the specific antigen have recently been shown to be potent inducers of antigen-specific Th1 responses conferring protection against infection with *L. major* (Ramirez-Pineda et al., 2004), this approach was selected in order to generate protective allergen-specific Th1 responses. First, the maturation status of BM-DC pulsed with CpG-ODN 1668 was verified. As already reported, BM-DC pulsed with CpG-ODN 1668 were able to upregulate the expression levels of costimulatory molecules and MHC class II, produce increased levels of proinflammatory cytokines and downmodulate the mRNA expression levels of the chemokine receptors CCR5 and CCR2, all features of fully mature DC (Ramirez-Pineda et al., 2004). Surprisingly, the application of BM-DC pulsed *ex vivo* with CpG/OVA one week before the initiation of the protocol to induce OVA-specific allergic Th2 responses in BALB/c mice (Fig. 15A) was unable to reduce the eosinophilia and the inflammatory response in the airways, in comparison to the application of either unpulsed BM-DC (Fig. 15B) or no cells (data not shown). The levels of OVA-specific IgG1 and IgE in the serum were also not modified by the application of CpG/OVA-pulsed BM-DC (Figure 15C). However, by using this approach, the production of OVA-specific IgG2a was greatly increased (Fig. 15C). Since the production of IgG2a is dependent on IFN-γ, this result suggests that the applied BM-DC were functional *in vivo* and induced OVA-specific Th1 responses.

To further evaluate the effect of the application of BM-DC pulsed with CpG/OVA on the generation of Th2 responses in comparison to the application of unpulsed BM-DC, the production of IL-4 and IL-5 in the supernatants of MLN cultures was measured after stimulation with anti-CD3/IL-2, OVA or medium alone. The application of CpG/OVA-pulsed BM-DC did not result in a significant decrease in the levels of IL-4 and IL-5 produced by MLN cells after stimulation with anti-CD3/IL-2 *in vitro* (Figure 15D). No cytokines were detected when the cells were cultured with OVA or in medium only.

These results clearly demonstrate that BM-DC pulsed with CpG/OVA induce Th1-type B cell responses but do not suppress the development of OVA-specific Th2 responses when applied intravenously into BALB/c mice before the initiation of the OVA/alum sensitization.

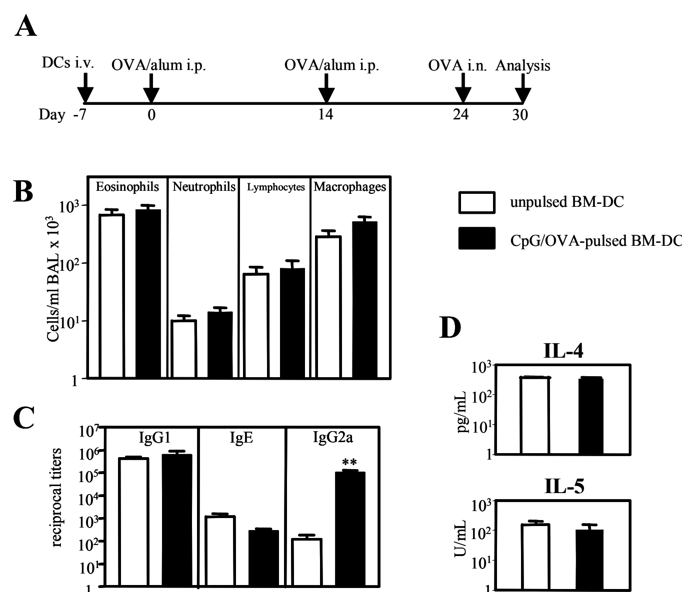

Figure 15. *Effect of the vaccination with CpG/OVA-pulsed BM-DC on the development of OVA-specific allergic Th2 responses.* BALB/c mice were vaccinated intravenously with CpG/OVA-pulsed BM-DC one week before the initiation of the OVA/alum sensitization protocol (**A**). Shown are the absolute numbers of the different cell types present in the BAL (**B**), the reciprocal titers of OVA-specific serum antibodies (**C**) and the cytokine levels detected in MLN cell cultures after stimulation with anti-CD3/IL-2 (**D**). Mean values of 5-7 mice/group ± SEM are shown. The experiment was repeated twice with similar results. **$p<0.01$ compared to the values obtained from mice injected with unpulsed BM-DC.

4.2.2. Intravenous injection of Langerhans cells pulsed with OVA does not reduce the development of OVA-induced Th2 responses in mice

As demonstrated above, BM-DC generated in the presence of high doses of GM-CSF were not able to protect BALB/c mice from the development of allergic-Th2 responses, after their maturation *ex vivo* in the presence of the allergen and CpG-ODN. Since these cells clearly show a myeloid phenotype ($CD11c^{+}$, $CD8\alpha^{-}$) (Ramirez-Pineda et al., 2004), other type of DC derived also from myeloid precursors, the Langerhans cells (LC), were selected to confirm the previous results with BM-DC. LC are a type of skin DC that constitutively express high levels of MHC class II molecules and are highly specialized in antigen presentation (Berberich et al., 2003). Furthermore, it was recently shown, that a similar approach using LC pulsed with defined Ag protected mice from developing lethal Th2 responses after infection with *L. major* (Berberich et al., 2003). For this purpose LC prepared from the ears of BALB/c mice and pulsed with OVA *ex vivo* were applied intravenously to naïve mice using the same protocol as for CpG/OVA-pulsed BM-DC (Fig. 15A).

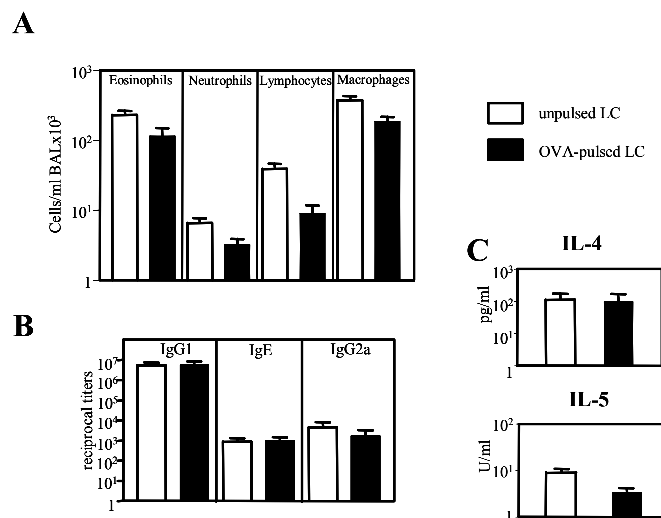

Figure 16. *OVA-specific Th2 responses in mice vaccinated with langerhans cells loaded with the allergen ex vivo.* BALB/c mice were vaccinated with OVA-pulsed LC and then subjected to the OVA sensitization protocol as shown in Figure 15A. Shown are the absolute numbers of the different cell types present in the BAL (**A**), the reciprocal titers of OVA-specific serum antibodies (**B**) and the cytokine levels detected in MLN cell cultures after stimulation with anti-CD3/IL-2 (**C**). Mean values of 5-7 mice/group ± SEM are shown. The experiment was repeated once with similar results.

Figures 16A and B show that the application of the OVA-pulsed LC also did not significantly suppress the airway eosinophilia or the levels of OVA-specific IgG1 or IgE in the serum in comparison to the application of unpulsed LC. In addition, the levels of IL-4 and IL-5 in cultures from MLN after stimulation with anti-CD3/IL-2 were also not reduced (Figure 16C). Thus, the previous results of this study using BM-DC maturated with CpG/OVA were confirmed by using LC loaded *ex vivo* with OVA. However, the application of LC pulsed with OVA did not lead to enhanced OVA-specific IgG2a levels.

4.2.3. Application of myeloid DC loaded *ex vivo* with OVA does not inhibit OVA-specific Th2 responses in C57BL/6 mice

Since the development of allergen-specific Th2-type responses in mice could be influenced by the genetic background of the strain (Morokata et al., 1999), I also evaluated whether the application of either CpG/OVA-pulsed BM-DC or OVA-pulsed LC decreased the development of OVA-induced Th2 responses in C57BL/6 mice. For this purpose, CpG/OVA-pulsed BM-DC or OVA-pulsed LC were prepared as described before and injected i.v. into C57BL/6 mice using the same protocol as for BALB/c mice (Figure 15A).

TABLE II. *OVA-specific allergic Th2 responses in C57BL/6 mice vaccinated i.v. with either CpG/OVA-pulsed BM-DC or OVA-pulsed LC*

		BM-DC		LC	
		Unpulsed BM-DC	**CpG/OVA-pulsed BM-DC**	**Unpulsed LC**	**OVA-pulsed LC**
Cells/ml BALx10^3	**Eosinophils**	227.8 ± 87.9	147.3 ± 27.2	299.7 ± 117.9	190.8 ± 71.4
	Neutrophils	4.8 ± 1.4	2.5 ± 0.5	5.0 ± 1.4	2.8 ± 0.9
	Lymphocytes	5.2 ± 1.3	2.5 ± 0.5	16.0 ± 4.3	11.0 ± 5.9
	Macrophages	94.5 ± 28.3	108.7 ± 21.6	161.0 ± 21.6	88.8 ± 21.5
OVA-specific Antibodies (reciprocal titers)	**IgG1 (x10^3)**	291.9 ± 101.6	478.1 ± 67.4	468.6 ± 131.9	656.9 ± 277.8
	IgE	232.3 ± 93.5	341.0 ± 174.8	898.2 ± 391.6	923.8 ± 280.7
	IgG2a	26.2 ± 6.9	14650.0 ± 6213.0**	25.1 ± 8.2	9.2 ± 4.0
Cytokines MLN	**IL-4 (pg/ml)**	596.4 ± 45.8	514.2 ± 26.3	n.d.	n.d.
	IL-5 (U/ml)	26.4 ± 1.2	12.3 ± 2.7*	28.9 ± 3.3	18.9 ± 2.5*

Mice were treated as described in Figure 15A. Shown are the mean values of 5-7 mice per group ± SEM. *p<0.05 and **p<0.01 compared to the values obtained in mice injected with unpulsed BM-DC or LC, respectively. n.d.: not detectable.

Table II shows that neither the application of BM-DC pulsed with CpG/OVA nor the application of LC pulsed with OVA resulted in a significant reduction of airway eosinophilia after the immunization with OVA/alum and the challenge with OVA. Also the levels of OVA-specific IgG1 and IgE in the serum were not altered by any of the myeloid DC-based vaccines. Again, an increase in the levels of serum OVA-specific IgG2a in the mice injected i.v. with CpG/OVA-pulsed BM-DC was observed. By using either mature BM-DC or LC loaded *ex vivo* with OVA intravenously, a significant decrease in the levels of IL-5 (but not IL-4) in the supernatants of MLN cultures from OVA-sensitized mice after stimulation with anti-CD3/IL-2 was observed (Table II). However, the decrease in the levels of IL-5 in the MLN after unspecific stimulation *in vitro* did not correlate with a reduction in the absolute numbers of eosinophils in the airways.

4.3. *Evaluation of NES as a new potential adjuvant to prevent the development of allergic responses in the murine model*

4.3.1. Application of NES together with OVA/alum reduces allergen-induced airway inflammation

Helminths produce substances that are able to downregulate allergic responses via immunregulatory mechanisms (Maizels et al., 2004). In particular, a recent study revealed that *N. brasiliensis* infection leads to a decrease in OVA-induced eosinophilia and eotaxin levels in the airways (Wohlleben et al., 2004). However, it is essential to isolate helminth-derived products with anti-allergy effects in order to design novel strategies to prevent allergic diseases. For this reason, I evaluated the capacity of NES, a excretory-secretory product derived from *N. brasiliensis* adult worms, to suppress OVA-specific allergic responses *in vivo*. NES was applied to C57BL/6 mice together with OVA/alum i.p. during the OVA

sensitization period and subsequently the induction of OVA-specific Th2 allergic responses was evaluated after the OVA i.n. challenge (Fig. 17A).

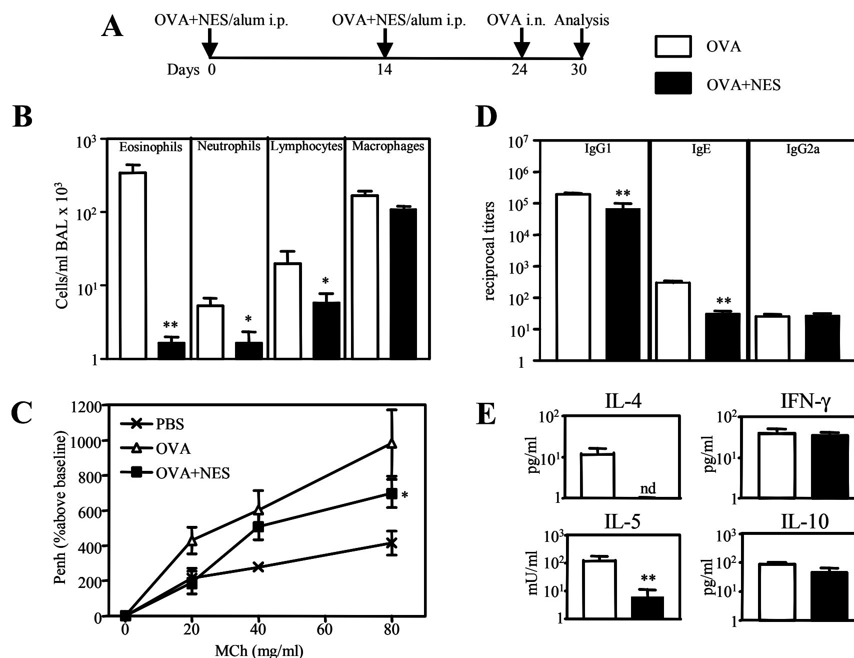

Figure 17. *Suppressive effect of NES on OVA-specific allergic Th2 responses.* Mice were vaccinated with NES during the OVA sensitization period (**A**). Depicted are the absolute numbers of the different cell types present in the BAL (**B**), the AHR in response to increasing concentration of methacoline (six intranasal challenges were applied in this particular experiment on days 24, 25, 26 and 31, 32, 33 and AHR was measured on day 34) (**C**), the reciprocal titers of OVA-specific serum antibodies (**D**) and the cytokine levels detected in the BAL fluid (**E**). Mean values of 6 mice/group ± SEM are shown. The experiment was repeated four times (with the exception of C) with similar results. *$p<0.05$ and **$p<0.01$ compared to the values obtained from mice sensitized only with OVA. The data shown in D were generously provided by Dr. Tobias Polte.

As shown in Figure 17B, the i.p. application of OVA+NES/alum was able to profoundly reduce the eosinophilia and the inflammatory response in the airways in comparison to the application of OVA/alum (200-fold reduction in the airway eosinophilia compared to OVA only treated mice). Moreover, the AHR after the application of high doses of methacholine was also significantly decreased in NES-vaccinated animals (Fig. 17C). In agreement with previously reported findings (Dainichi et al., 2001), NES was able to significantly suppress the OVA-specific IgG1 and IgE serum levels although the production of OVA-specific IgG2a was not affected (Fig. 17D). As the production of IgG2a is stimulated by IFN-γ, these results suggest that the mechanism by which NES is downregulating OVA-specific Th2 responses is

not related to the induction of OVA-specific Th1 responses *in vivo*. This assumption was further supported by the observation that no variation in the levels of IFN-γ was detected in the BAL fluid by ELISA as shown in Figure 17E. Likewise, levels of IL-10 detected in the BAL fluid were not altered by the application of NES (Fig. 17E). Nevertheless, the levels of IL-4 and IL-5 in the BAL fluid were significantly reduced after NES vaccination (Fig. 17E).

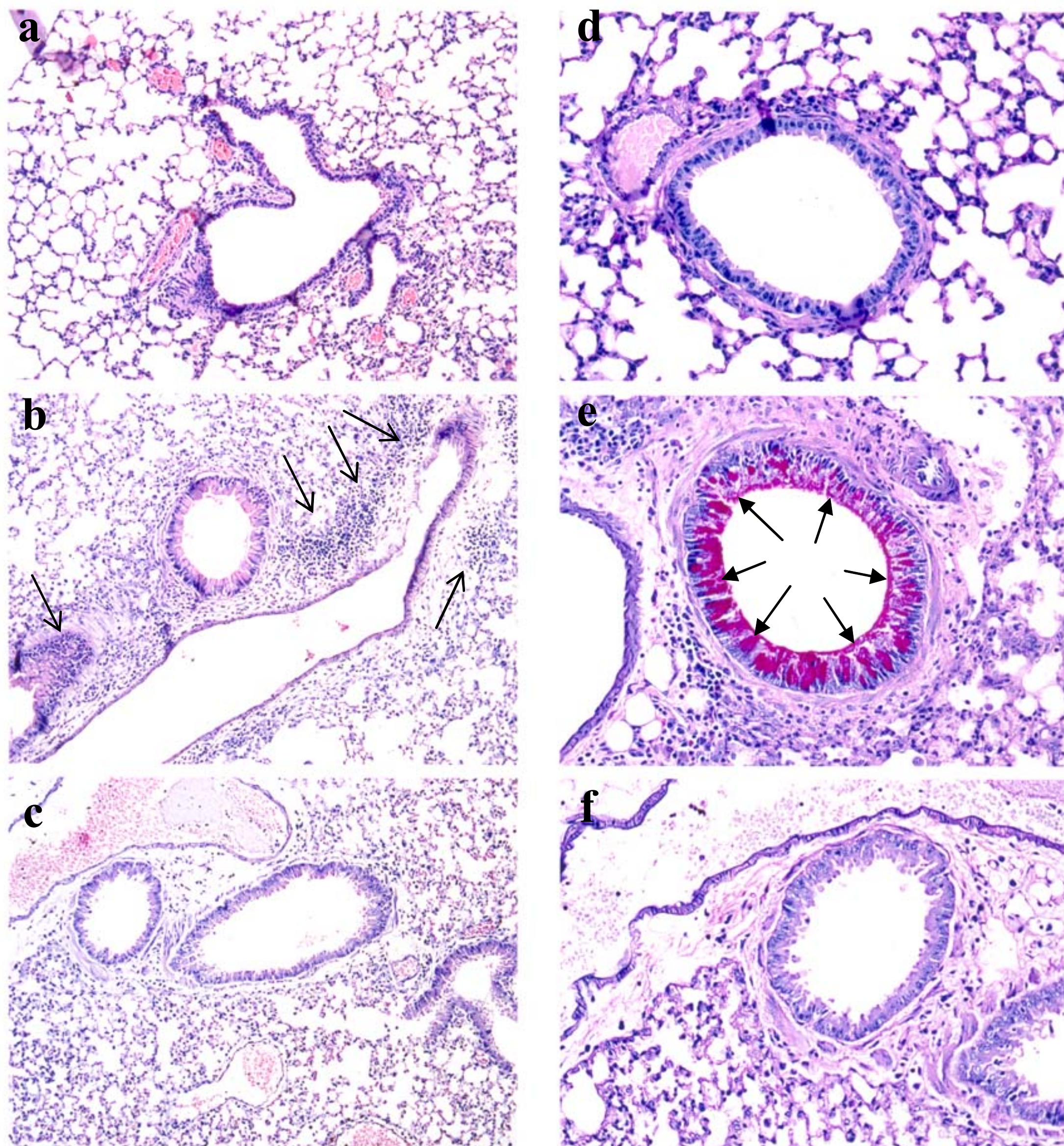

Figure 18. *Effect of NES on the development of airway inflammation and goblet cell metaplasia after OVA sensitization and challenge.* Mice were vaccinated as described in Figure 17A. Shown are lung sections subjected to H&E (a-c) or PAS (d-f) staining from PBS- (a, d), OVA- (b, e) and OVA+NES- (c, f) immunized mice. Representative examples of six mice/group are shown (a-c, 20x and d-f, 200x magnification). Open arrows indicate areas with peribronchial and perivascular inflammation, closed arrows indicate goblet cell metaplasia.

Evaluation of histopathology sections of the lung revealed a strong reduction in the perivascular and peribronchial inflammation by using OVA+NES/alum in comparison with OVA/alum (mean scores: PBS: 0, OVA/alum: 2, OVA+NES/alum: 0, Fig 18a-c). In addition, mice vaccinated with NES exhibited also a complete absence of goblet cell metaplasia in the airways (mean scores: PBS: 0, OVA/alum: 3, OVA+NES/alum: 0, Fig 18d-f).

4.3.2. LPS contamination is not responsible for the suppressive effect of NES on the development of OVA-specific Th2 responses

As already explained, NES was obtained from *N. brasiliensis*-adult worms that were isolated from the gut of infected rats. Even though the worms were repeatedly washed in sterile saline and incubated with medium containing high concentration of antibiotics, traces amounts of bacterial LPS contaminating NES were always detected by the Limulus assay (<1 μg/ml).

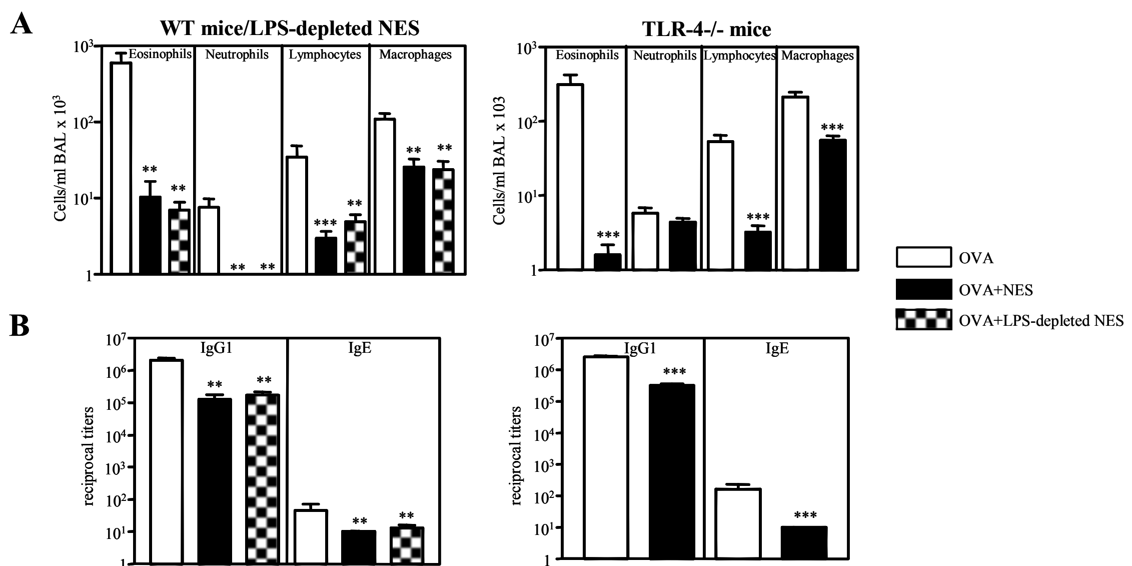

Figure 19. *NES-mediated suppression of OVA-specific allergic responses in the absence of either LPS or LPS-mediated signaling.* Depicted are the absolute numbers of the different cell types present in the BAL (**A**) and the OVA-specific IgG1 and IgE serum titers (**B**) of mice by using either LPS-depleted NES in wild type mice or alternatively, LPS-contaminated NES in TLR-4-deficient mice. Mean values of 6-7 mice/group ± SEM are shown. **$p<0.01$ and ***$p<0.001$ compared to the values obtained from mice sensitized only with OVA.

As LPS has been shown to exert anti-allergy effects under certain circumstances (Tulic et al., 2000), it was next evaluated if NES could still reduce OVA-induced allergic responses in the absence of LPS contamination. For this reason LPS-depleted NES was prepared and its capacity to downmodulate OVA-induced allergic responses in mice was investigated. As shown in Figure 19, LPS-depleted NES was comparable to the normal NES (contaminated with traces of LPS) in its ability to reduce the airway eosinophilia and the serum levels of OVA-specific IgG1 and IgE in wild type mice when applied together with OVA/alum during the OVA-sensitization period. Further experiments in TLR-4-deficient mice confirmed that NES could still downregulate OVA-specific allergic responses even in the absence of LPS-mediated signaling (Fig. 19).

4.3.3. The absence of IFN-γ does not affect the ability of NES to downmodulate OVA-specific Th2 responses

In the previous experiments, no evidence suggested that an IFN-γ-dependent mechanism were responsible for the ability of NES to suppress OVA-induced allergic Th2 responses *in vivo*. Since IFN-γ is one of the main cytokines secreted by Th1 cells, the absence of increased serum levels of OVA-specific IgG2a (IFN-γ-stimulated) (Fig. 17D) or IFN-γ in the BAL of NES-vaccinated mice (Fig. 17E) argued against the possibility that OVA-specific protective Th1 responses have been induced by the application of NES. However, IFN-γ has been widely shown to be able to prevent the generation of Th2 responses both *in vitro* and *in vivo* (Gajewski and Fitch, 1988) (Parronchi et al., 1992) (Lack et al., 1996) (Li et al., 1996). In addition, previous results of this thesis showed that Th1-inducing adjuvants strongly reduce the development of Th2 allergic responses in mice mainly via an IFN-γ-dependent mechanism (see Fig. 4 and 5). Thus, to definitively verify that IFN-γ plays no role in the ability of NES to downregulate OVA-induced allergic responses, either OVA/alum or OVA+NES/alum were

applied to IFN-γ-deficient mice following the protocol illustrated in Figure 17A. However, the absence of IFN-γ did not affect the suppressive capacity of NES on the eosinophilia and the inflammatory response in the airways (Fig. 20A). Also IFN-γ-deficient NES-vaccinated mice showed reduced levels of OVA-specific IgG1 and IgE in the serum in comparison to mice injected only with OVA/alum (Fig. 20B). These results demonstrate that the generation of OVA-specific Th1 cells producing IFN-γ is not the mechanism responsible for the ability of NES to downregulate OVA-induced Th2 responses in mice.

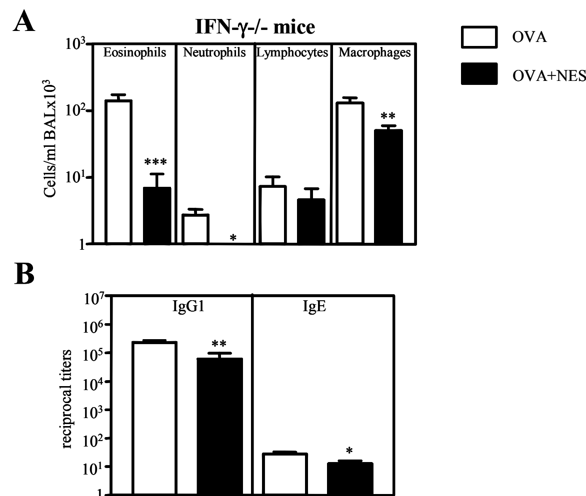

Figure 20. *Suppressive effect of NES on OVA-specific allergic responses in the absence of IFN-γ.* IFN-γ-deficient mice were vaccinated with OVA+NES using the same protocol described in Figure 17A. Shown are the absolute numbers of the different cell types present in the BAL (**A**) and the OVA-specific IgG1 and IgE serum titers (**B**). Mean values of 5-6 mice/group ± SEM from two separate experiments are shown. *p<0.05, **p<0.01 and ***p<0.001 compared to the values obtained from mice sensitized only with OVA.

4.3.4. The ability of NES to downmodulate OVA-induced Th2 responses is independent upon IL-10

Numerous reports have shown that IL-10-producing Tr cells play an important role in the ability of the helminth or helminth-derived components to prevent allergic responses in humans and mice (Biggelaar et al., 2000) (Kleij et al., 2002) (Maizels et al., 2004). Moreover, the infection with *N. brasiliensis* seems to suppress the development of allergen-induced

airway eosinophilia in mice via a mechanism dependent upon IL-10 (Wohlleben et al., 2004). For this reason, I evaluated whether NES was still able to suppress OVA-induced allergic responses in the absence of IL-10. Monoclonal blocking antibodies against the IL-10-receptor (IL-10R Ab) were applied i.p. and simultaneously, the protocol to induce OVA-specific Th2 responses using either OVA/alum or OVA+NES/alum was initiated in mice as described in *Material and Methods*. Mice injected i.p. with either PBS or an isotype-matched antibody were used as controls. Surprisingly, mice vaccinated with NES could significantly suppress airway eosinophilia and inflammatory response in the airways and decrease OVA-specific IgG1 and IgE in the serum after the blocking of IL-10-mediated signalling using IL-10R Ab (Fig. 21A). The application of isotype-matched control antibody (Ctrl Ab) had no effect on the amounts of eosinophils recruited into the airways or the levels of OVA-specific IgG1 and IgE in the serum of mice receiving either OVA/alum or OVA+NES/alum in comparison to the application of PBS (Fig. 21A).

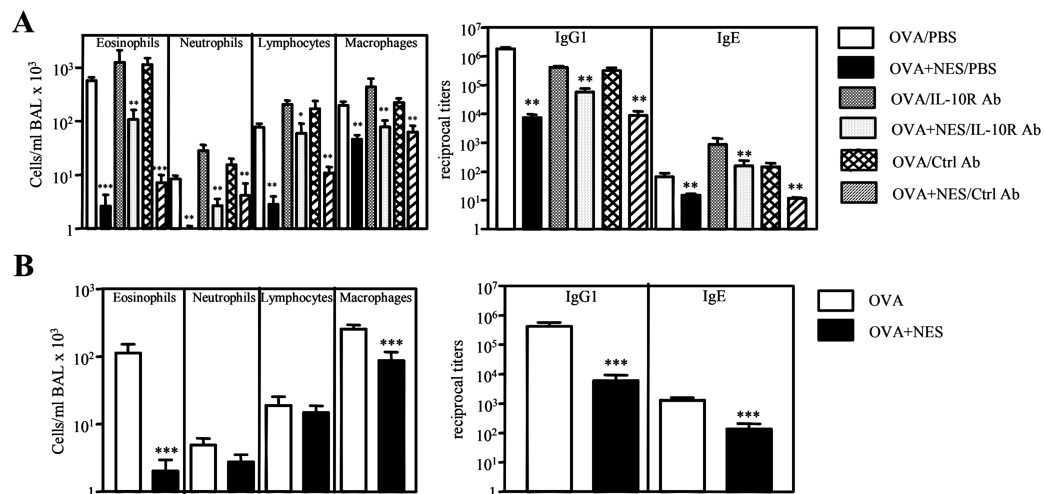

Figure 21. *Suppressive effect of NES on OVA-specific allergic responses in the absence of IL-10.* The downregulatory effect of NES on OVA-specific allergic Th2 responses was evaluated either by blocking IL-10-mediated signaling treating the mice with IL-10R antibodies as described in *Materials and Methods* (3-6 mice/group) (**A**) or in IL-10-deficient mice as described in Figure 17A (6-7 mice/group) (**B**). Depicted are the absolute numbers of the different cell types present in the BAL and the OVA-specific IgG1 and IgE serum titers. Mean values ± SEM are shown. *p<0.05, **p<0.01 and ***p<0.001 compared to the values obtained from mice sensitized only with OVA.

To further confirm these results, IL-10-deficient mice were subjected to the NES application following the protocol shown in Figure 17A. As shown in Figure 21B, also in the absence of IL-10, NES is able to decrease OVA-induced allergic responses in mice. These data suggest that NES reduces OVA-induced Th2 responses *in vivo* in an IL-10-independent manner.

4.3.5. Non-protein components of NES mediate its suppressive effect on the OVA-induced allergic responses

In an attempt to elucidate the components of NES responsible for its ability to downmodulate OVA-specific allergic responses, NES was treated with proteinase K beads. As revealed by a silver staining of a polyacrylamide gel (which is a highly sensitive method detecting nanogram quantities of proteins), the treatment with proteinase K beads induced complete digestion of all NES protein components (Fig. 22A). The capacity of proteinase K-digested NES to decrease OVA-specific allergic responses in mice was subsequently compared with that of non-digested NES. As shown in Figure 22B, proteinase K-digested NES retained the ability to reduce OVA-induced airway eosinophilia and the inflammatory response in the airways. As expected, the effect induced by proteinase K-digested NES was not as great as the effect induced by non-digested NES to suppress airway eosinophilia suggesting that some protein components of NES may also decrease OVA-specific allergic responses *in vivo*. However, the suppressive capacity of the proteinase-K-digested NES on the airway eosinophilia was still significantly pronounced (20-fold reduction compared to OVA only treated mice). Moreover, proteinase K-digested NES could also significantly reduce the production of OVA-specific IgG1 and IgE to levels comparable to those obtained after vaccination with non-digested NES (Fig. 22C).

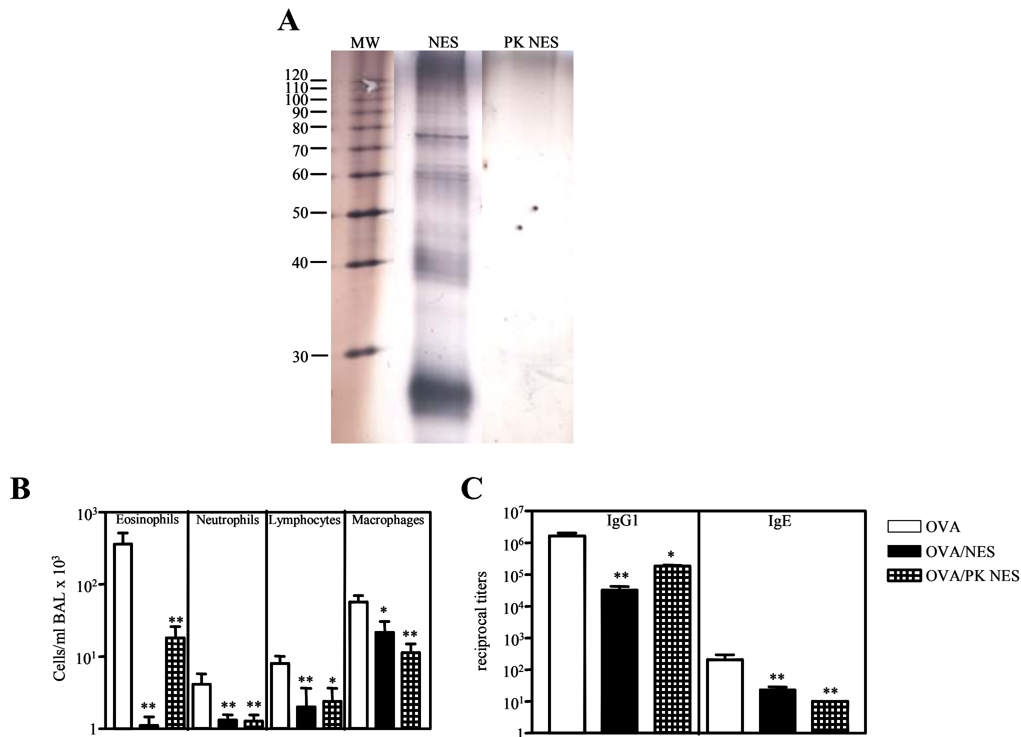

Figure 22. *Effect of the vaccination with proteinase K-digested NES on OVA-specific allergic Th2 responses.* The ability of non-protein components of NES to reduce OVA-specific allergic responses was evaluated by applying proteinase K-digested NES (PK NES) using the protocol described in Figure 17A. The digestion of proteins by proteinase K was controlled by performing a silver staining of a polyacrylamide gel (**A**). Depicted are the absolute numbers of the different cell types present in the BAL (**B**) and the OVA-specific IgG1 and IgE serum titers (**C**). Mean values of 5-6 mice/group ± SEM are shown. The experiment was repeated once with similar results. *p<0.05 and **p<0.01 compared to the values obtained from mice sensitized only with OVA.

I additionally performed experiments denaturating the NES proteins by heating this product at 100°C during 20 min. Heat-treated NES was applied to the mice together with OVA/alum as indicated in figure 17A and the inflammatory response in the airways was evaluated after the OVA challenge. Remarkably, heat-treated NES was still able to significantly decrease airway eosinophilia (OVA: 361.7±158.2 vs OVA+heat-treated NES: 0.3±0.2** eosinophils/ml BALx10^3; mean of 6 mice/group± SEM representative of two separate experiments; **p<0.01).

Thus, these observations demonstrate that *N. brasiliensis* adult worms secrete non protein components or alternatively heat-resistant proteins which exhibit anti-allergy properties *in vivo*.

4.3.6. NES suppresses OVA-specific Th2 responses *in vivo* in the presence of a strong NES-specific Th2 environment

In the present study, it has been demonstrated that NES has the ability to downregulate OVA-specific Th2 responses in mice via a mechanism independent upon IFN-γ and IL-10 and that these effects are partially attributed to non-protein components of NES. However, previous studies have shown that this product drives strong NES-specific Th2 immune responses in mice (Holland et al., 2000) (Balic et al., 2004) (Holland et al., 2005). In order to confirm the Th2-promoting activity of NES, mice were immunized with OVA+NES/alum as described before (Fig. 17A) and then, the recruitment of eosinophils and other inflammatory cells into the airways (indicative of Th2-mediated inflammation) after the intranasal application of NES (instead of OVA) was evaluated. As shown in Figure 23A, although NES is able to suppress eosinophilia and inflammatory response in the airways after OVA i.n. challenge, the intranasal application of NES instead of OVA after sensitization with OVA+NES/alum i.p. is able to induce airway eosinophilia and inflammation to levels comparable to those induced by the application of OVA i.n. after sensitization with OVA/alum i.p..

These results were confirmed by analyzing the percentage and absolute numbers of IL-5-producing $CD4^+$ cells present in the BAL of treated animals (Fig. 23B and C). The application of OVA i.n. after sensitization with OVA+NES/alum (OVA+NES/alum-OVA i.n. in the figure) is able to strongly decrease the percentage and absolute numbers of IL-5-producing CD4+ cells in the BAL fluid in comparison to the application of OVA i.n. after sensitization with only OVA/alum (OVA/alum-OVA i.n. in the figure). On the contrary, when NES was used for the intranasal challenge after sensitization with OVA+NES/alum (OVA+NES/alum-

NES i.n. in the figure), increasing numbers of IL-5-producing CD4+ cells could be detected in the BAL.

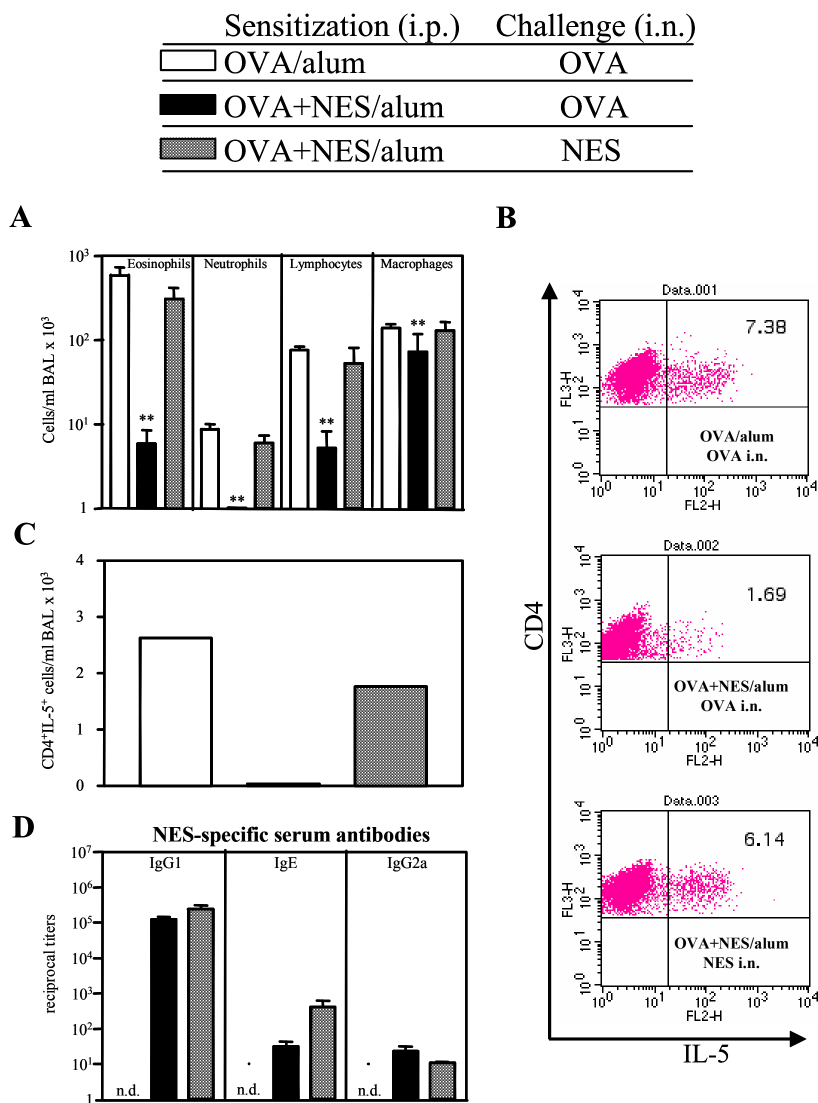

Figure 23. *Capacity of NES to induce specific Th2 responses.* Mice were vaccinated using the protocol described in Figure 17A. Where indicated, NES was applied during the intranasal challenge instead of OVA. Shown are the absolute numbers of the different cell types in the BAL (**A**), the percentages (**B**) and absolute numbers (**C**) of IL-5-producing CD4+ T cells in pooled cells from the BAL and the reciprocal titers of NES-specific serum antibodies (**D**). Mean values of 5-6 mice/group ± SEM are shown. *p<0.05 and **p<0.01 compared to the values obtained from mice sensitized and challenged only with OVA.

The production of NES-specific serum antibodies was also evaluated in these animals (Fig. 23D). The application of NES i.p. and i.n. significantly enhanced the levels of NES-specific IgG1 and IgE in the serum. The production of NES-specific IgG2a was not affected by using

this treatment. Taken together, these results prove that NES decreases OVA-specific allergic responses in the presence of a strong NES-specific Th2 environment.

5. DISCUSSION

Allergic diseases and in particular allergic asthma are considered to be a tremendous health and economic burden for the society especially in the last decades (Aberg et al., 1995) (Hartert and Peebles, 2000) (Akinbami and Schoendorf, 2002) (Devenny et al., 2004). With the decrease in lung function, costs due to asthma treatment have risen considerably (Rodrigo et al., 2004). Although much progress has been made in understanding the immunological mechanisms underlying the development of allergic responses, alternatives for the development of a safe and effective anti-allergy vaccine remain elusive. However, many epidemiological and experimental studies suggest that previous exposure to certain microorganisms or microorganism-derived products decreases the incidence and severity of allergic diseases (Trujillo and Erb, 2003) (Maizels et al., 2004). These evidences have resulted in the so-called "hygiene hypothesis" which proposes that infectious agents might influence the development allergic diseases through the induction Th1 or Tr immune responses (Wills-Karp et al., 2001) (Rook and Brunet, 2002). The hygiene hypothesis represents the immunological basis of the present study, which attempted to identify promising vaccine candidates against allergic asthma in the murine model. Components derived from intracellular bacteria (BCG) as well as helminth-derived products were evaluated. In addition, novel vaccination strategies such as the use of dendritic cells pulsed with a microorganism-derived component (CpG) as allergen carriers, were also investigated.

First of all, the inhibitory effects on Th2 cell development of several known Th1-inducing adjuvants (i.e. live BCG, hk-BCG, CpG and PPD) as components of anti-allergy vaccine candidates were compared. Numerous studies have shown that the induction of allergen-specific or unspecific Th1 responses are associated with the inhibition of allergic Th2

responses in the airways (Erb and Wohlleben, 2002). Several epidemiological studies also indicate that this may be true in humans (Trujillo and Erb, 2003). In order to induce Th1 responses, different approaches have been used. The most effective ones include live or dead bacteria, bacterial products such as LPS, MPL (monophosphoryl lipid A, a detoxified derivative of LPS), PPD or CpG-ODN and DNA vaccines (Erb et al., 1998) (Kline et al., 1998) (Tulic et al., 2000) (Hansen et al., 2000) (Wheeler et al., 2001) (Horner et al., 2001) (Hattori et al., 2002). However, it is not clear which approach is the best and safest, because although Th1 responses are very effective in inhibiting Th2 responses in mice, they harbor the danger of Th1 cell mediated pathology in the lung (Erb and Wohlleben, 2002).

When comparing live BCG, hk-BCG, CpG and PPD, this study showed that all the different tested adjuvants reduced the development of allergic inflammation in the airways, including eosinophilia and mucus production when they were applied together with the allergen during the i.p. sensitization. The strongest effects were induced by live BCG and hk-BCG. The finding that live BCG also reduced the development of allergen-specific IgG1 and IgE serum levels could be expected since this application was the most efficient in reducing Th2 responses in the lung. However, the observation that the application of hk-BCG or CpG-ODN did not reduce the amounts of OVA-specific IgE or IgG1 in the serum was surprising, since in both cases increased OVA-specific IgG2a levels were found, clearly demonstrating that hk-BCG and CpG-ODN had effects on B cell isotype switching towards Th1 responses during the immunization. Another unexpected finding was that the application of PPD, which did not increase allergen-specific IgG2a levels, reduced the serum levels of OVA-specific IgG1 and IgE. Recently Rha et al. reported that the application of heat shock proteins (hsp) from *M. leprae* also reduced the development of allergen-specific serum IgE levels (Rha et al., 2002). It may be possible that the PPD from *M. tuberculosis* that was used in the present study, contains hsp, which mediate the suppressive effect on IgE production by B cells. However the

same authors also reported that hsp from BCG or *M. tuberculosis* did not have this effect. Currently we have no explanation on how the addition of PPD to OVA/alum mediates the decrease in IgE and IgG1 production, without affecting OVA-specific IgG2a levels in the serum. However, our observation is supported by results from Hattori et al. which also observed that the application of PPD reduced the induction of *Schistosoma mansoni* egg antigen (SEA)-specific IgG1 and IgE titers in mice (Hattori et al., 2002).

The suppressive effects of the different adjuvants, with the exception of PPD, on the development of allergen-induced Th2 responses in the lung correlated with decreased amounts of IL-5, increased amounts of IFN-γ in the airways and increased levels of allergen-specific IgG2a levels in the serum. Furthermore, higher amounts of Th1 cell and decreased amounts of Th2 cell numbers were detected in the BAL fluid of the vaccinated mice. This suggests that the inhibitory effects on Th2 responses were dependent upon the induction of Th1 responses. This hypothesis was confirmed by repeating the experiments in IFN-γ- and IL-12p35-deficient mice. Taken together, the strong reduction in airway eosinophilia observed in mice immunized with OVA together with live BCG, hk-BCG or CpG-ODN may be explained by a reduction in Th2 cell development induced by Th1 cells. Interestingly, live BCG in contrast to hk-BCG, CpG-ODN or PPD still decreased Th2 responses in the lung independent of IFN-γ or IL-12, albeit to a lower degree than the effect observed in wild type mice. Additionally, the suppressive effect of live BCG on allergen-induced airway eosinophilia was still observed in IL-12p35/IL-12p40-double deficient mice. Thus, in addition to IFN-γ, IL-12 and IL-23, which consists of one IL-12p40 subunit (Trinchieri et al., 2003), other factors appear to play a role in the strong inhibition of allergic Th2 responses mediated by live BCG.

There are several explanations for these findings. First, it has been reported that IL-12-independent Th1 development could be induced by the cooperative action of IL-18 and other

cytokines such as IFN-α or IL-1α/β (Takeda et al., 1998). In addition, Lewkowich et al. showed that although IFN-γ is a key negative regulator of the Th2 immune response, IL-18 also negatively regulates $CD4^+$ T cell-derived IL-4 synthesis via an IFN-γ independent mechanism (Lewkowich and HayGlass, 2002). Furthermore, IL-27 a heterodimeric cytokine produced by APC, which synergizes with IL-12 to drive Th1 cell development, has also been shown to inhibit the expression of GATA-3 (Das et al., 2001) (Pflanz et al., 2002) (Lucas et al., 2003). Since GATA-3 is required for the development of Th2 cells (Zheng and Flavell, 1997), it may be possible that live BCG induces the expression of IL-27, thereby leading to the suppression of Th2 responses in the absence of IFN-γ. Taken together these reports may explain how in the absence of IFN-γ and IL-12, live BCG suppresses Th2-mediated allergic inflammation.

A further explanation for these findings might be that live BCG induces the development of Tr cells, which then mediate the suppression of Th2 responses. Recently, it has been shown that the suppression of airway eosinophilia by killed *M. vaccae* is mediated by allergen-specific Tr cells and dependent upon IL-10 and TGF-β (Zuany-Amorim et al., 2002). Therefore, it is possible that the application of live BCG may also induce Tr cells, which may in addition to Th1 responses contribute to the inhibition of Th2 responses. Supporting this view was the finding that live BCG induced the secretion of large amounts of IL-10 by BM-DC. However, the experiments using IL-10-deficient mice indicated that the lack of IL-10 does not abolish the suppressive effect of live BCG on Th2 responses in the presence of IFN-γ. It may be possible that IL-10 plays a more important role in inhibiting allergic responses under conditions where IFN-γ is lacking or more weakly induced than after the application of live BCG (possibly during the application of killed *M. vaccae*). Alternatively, in the absence of IFN-γ, Tr cells producing TGF-β or Tr cells inhibiting Th2 cell proliferation through a cell-

cell contact mediated mechanism may be responsible for the observed suppression of allergen-induced Th2 responses, independent of IL-10 (Robinson et al., 2004).

The inhibitory effect of live BCG on the development of allergic Th2 responses was also independent upon TLR-2-, TLR-4- or MyD88-mediated signaling as observed in TLR-2/TLR-4-double deficient mice and MyD88-deficient mice. This finding is in agreement with previous reports which demonstrate that even in the absence of TLR-2, TLR-4 or MyD88, systemic Th1 development with high IFN-γ production by splenocytes could be stimulated after the application of live BCG inducing bacterial clearance (Heldwein et al., 2003) (Nicolle et al., 2004a) (Nicolle et al., 2004b). The *in vitro* data also indicate that particulate or soluble fractions of BCG signal mainly through TLR-2, whereas live BCG triggers other pathways which may be linked to the phagocytosis process (Uehori et al., 2003). Indeed, *M. bovis* BCG can bind to and enter macrophages through many other mechanisms including the ligation to complement receptors, mannose receptors, scavenger receptors or the opsonization with C-type lectins like surfactant-associated protein A (SP-A) (Ernst, 1998) (Laan et al., 1999) (Weikert et al., 2000). In addition, it has been shown that DC-SIGN (DC-specific ICAM-3-grabbing nonintegrin) is an specific dendritic cell receptor (also a C-type lectin) which captures and internalizes intact BCG bacteria through interaction with ManLAM (Geijtenbeek et al., 2003). These evidences suggest that redundant mechanisms contribute to the uptake of *M. bovis*-BCG by antigen presenting cells inducing the generation of efficient immune responses during the infection therefore retaining the ability of these bacteria to inhibit the development of Th2-mediated allergic responses.

The experiments presented here clearly show that the application of the different adjuvants simultaneously with the allergen inhibit the development of Th2 responses in the lung. These findings support the hypothesis that infectious diseases or the exposure to pathogen-derived

products, which induce Th1 responses, help in inhibiting the development of asthma in humans when the Th1 inducing agents are present during allergen-sensitization. A further goal of our studies was to ascertain whether our vaccination candidates were also capable to inhibit Th2 responses after a second round of allergen-sensitization in the absence of any of the Th1-inducing adjuvants. Here, it was found that the application of live or hk-BCG but not CpG-ODN or PPD was able to reduce allergen-induced Th2 responses in the lung after a second round of OVA/alum sensitization. This effect was long lasting, since airway eosinophilia was still significantly reduced when the second round of OVA sensitization started 12 weeks after the vaccination. Furthermore, suppression of airway eosinophilia could be transferred to naive mice by $CD4^+$ T cells purified from the spleen of immunized mice, clearly demonstrating that the effect was induced by this subpopulation of cells. The suppression of airway eosinophilia correlated with decreased numbers of Th2 cells present in the airways. A recent study indicates that infection with influenza A virus reduces the development of airway eosinophilia by interfering with the recruitment of Th2 cells into the airways and that this effect was associated with increased levels of IFN-γ and IFN-γ-producing cells in the airways (Wohlleben et al., 2003). It is tempting to speculate that the suppression of allergen-induced airway eosinophilia and mucus production observed in the live BCG-vaccinated mice may be due to the presence of Th1 cells secreting IFN-γ in the lung, which results in the inefficient recruitment of allergen-specific Th2 cells into the airways. Supporting this view was the finding that vaccination with OVA+live BCG/alum prior to the application of *in vitro* generated OVA-specific Th2 cells intravenously, reduced the recruitment of these cells into the airways in comparison to OVA/alum only immunized mice.

Another interesting finding was that although live BCG was the most efficient component of a vaccine protecting against the development of allergen-induced airway inflammation, CpG-

ODN was more efficient than live BCG in inducing the production of OVA-specific IgG2a during the primary sensitization with OVA. This observation suggests that the application of CpG-ODN together with OVA also induces strong allergen-specific Th1 responses. Supporting this view was our finding that CpG-ODN was also more efficient than live BCG to induce the secretion of IL-12p70, an important cytokine mediating Th1 cell development, by BM-DC after *in vitro* stimulation. This implies that the strength of the allergen-specific Th1-type B cell response, induced after vaccination, is not a decisive factor, which determines how strong the suppressive effect on Th2 responses will be. It can be possible, that similar to killed *M. vaccae* (Zuany-Amorim et al., 2002), live BCG may also induce the development of Tr cells, which in addition to Th1 cells contribute to the reduction of Th2 responses as already discussed.

The results of this study show that mice are efficiently protected from developing airway inflammation and AHR when live BCG was used as a vaccine adjuvant. This suggests that an anti-asthma vaccine using live BCG may also be effective in humans. However, the data also show that the protective effects were mediated to a large extent by Th1 cells. Since Th1 cells are powerful mediators of inflammation, it is important to rule out that a vaccine aimed at protecting humans from asthma utilizing allergen-specific Th1 responses does not lead to pulmonary Th1 cell mediated inflammation and tissue destruction. None of the experiments performed in this study provided evidence that it was occurring in mice vaccinated with the different Th1 inducing adjuvants and i.n. challenged once with OVA. However, when mice were vaccinated with OVA+live BCG/alum and then repetitively exposed to OVA i.n., some degree of Th1 type inflammation was detected in the airways. This indicates that a vaccine inducing Th1 responses may lead to allergen-induced Th1 inflammation in the airways as previously suggested (Hansen et al., 1999) (Randolph et al., 1999).

In conclusion, the experiments show that all the tested Th1-inducing adjuvants had suppressive effects on the development on allergen-induced Th2 responses (live BCG>>hk-BCG>CpG-ODN>PPD), which were to a great extent associated with the presence of IFN-γ or IL-12. However, the degree of suppression and the type of Th2 response (airway inflammation versus the production of allergen-specific IgE and IgG1) were dependent upon the adjuvant used and how it was applied. In general, it was found that pulmonary Th2 responses were more effectively inhibited by the addition of the adjuvants than the production of allergen-specific IgG1 and IgE. The reason for this important observation is unclear since the induction of allergen-induced airway eosinophilia and the development of B cells secreting allergen-specific IgG1 and IgE are dependent upon the presence of allergen-specific Th2 cells. This study also clearly shows that live BCG was more efficient than hk-BCG, CpG-ODN or PPD as an adjuvant for an anti-asthma vaccine. However, it can be possible that using higher amounts of hk-BCG, CpG-ODN or PPD, or applying them more frequently may yield different results. The repeated application of for example, CpG-ODN may lead to unwanted side effects, as recently reported by Heikenwalder et al. (Heikenwalder et al., 2004) The findings of this study may contribute to the design of an efficient and safe vaccine protecting humans from developing allergic disorders in the future.

The second aim of my thesis was to develop a DC-based vaccination strategy also leading to the induction of allergen-specific Th1 cells which in turn should protect mice from developing allergen-specific Th2 responses. Two different approaches were used: the intravenous application of either CpG/allergen-pulsed BM-DC or allergen-pulsed LC. However, both approaches failed to reduce allergic airway inflammation or the production of allergen-specific IgG1 or IgE in the serum in two different strains of mice (BALB/c and C57BL/6) when DC were applied before the initiation of the OVA/alum sensitization. Surprisingly, by using an identical protocol, others have previously found that *L. major*-

induced Th2 responses could be inhibited by vaccination with CpG-treated BM-DC or LC pulsed with *L. major* lysate (Berberich et al., 2003) (Ramirez-Pineda et al., 2004). This result suggest that the type of antigen used may be of importance when applying DC-based vaccination protocols. Based on a recent report (Heer et al., 2004), it can also be postulated that the type of DC is decisive for the efficacy of these approaches. This report showed that different subsets of DC in the lung govern the induction of tolerance versus immunity to inhaled allergens. Whereas myeloid DC were important for generating T-cell division and priming, plasmocytoid DC (pDC) suppressed T cell effector functions. As the DC subsets used in this study (BM-DC and LC) were all derived from myeloid lineage, it is possible that they can induce Th1 cell development but that they do not have the ability to efficiently suppress the development of allergen-specific Th2 cells. This assumption is supported by the observation in this study that CpG/OVA-pulsed BM-DC failed to reduce allergic Th2 responses although they induced increasing levels of serum OVA-specific IgG2a, indicative of allergen-specific Th1-cell development.

On the other hand, the results of the present study are in contrast to previously published work. It was reported that the activation of peroxisome proliferator-activated receptor γ (PPAR-γ, an array of receptors that are activated by polyunsaturated fatty acid derivatives or phospholipids) in allergen-pulsed BM-DC leads to decreased airway eosinophilic inflammation in BALB/c mice treated with these DC (Hammad et al., 2004). Also, the application of myeloid DC stimulated with LPS together with the allergen have also been reported to suppress airway Th2 responses in mice (Kuipers et al., 2003). Furthermore, a study conducted by Chiang et al. showed that BM-DC pulsed with either the guanosine analogue ribavirin or CpG (CpG 1826) together with OVA could downregulate OVA-specific Th2-type immune responses and alleviate airway inflammation in BALB/c mice (Chiang et al., 2003). A possible reason for the apparent discrepancy between the results from the present

study and previously published findings may be that the immunization protocol used here (sensitization of mice with OVA and alum) induces much stronger allergen-specific Th2 responses than the protocols used by other investigators (e.g. sensitization of mice with OVA without alum). Supporting this view are other studies showing that a protocol used to inhibit allergic asthma in mice produced positive results in a mild but not the severe asthma model (Schmitz et al., 2003) (Deurloo et al., 2001).

Taken together, my results show that vaccination with either BM-DC activated with CpG and pulsed with OVA or LC only pulsed with OVA one week before the sensitization with the allergen, did not reduce the development of allergen-specific Th2 responses in mice. It is possible that applying the CpG/OVA pulsed BM-DC or OVA pulsed LC via a different route or more often may yield more promising results.

In the last part of the present study, the ability of NES, a helminth-derived component, to inhibit OVA-induced Th2 responses was evaluated in the murine model. Interestingly, NES was highly effective in suppressing eosinophilia, AHR, mucus production and inflammatory responses in the airways when applied together with OVA/alum during the sensitization period. By using LPS-depleted NES, it was also clearly proved here that NES-mediated suppression of OVA-specific Th2 responses was not due to the traces of LPS contamination in the NES preparations. Moreover, the use of TLR-4-deficient mice, which have impaired LPS-mediated signalling, confirmed these results. On the other hand, due to the accumulating evidence indicating that Th1-inducing adjuvants efficiently downmodulate allergen-specific Th2 responses (Trujillo and Erb, 2003), I also investigated whether the suppressive effect of NES on OVA-specific allergic responses was mediated by the generation of allergen-specific Th1 responses. This hypothesis seems unlikely considering that helminth or helminth-derived products are the classic example of Th2-cell inducers in humans and experimental models

(Maizels and Yazdanbakhsh, 2003). Most strikingly, NES has been widely presented as a potent Th2-inducing adjuvant (Holland et al., 2000) (Balic et al., 2004) (Holland et al., 2005). However, there are several interesting exceptions to this rule of Th2-cell skewing by helminths. For example, the initial infective stages of schistosomes (cercariae) stimulate Th1-cell responses in the mouse model. Only as infection progresses, the response switches to Th2, driven by schistosome-egg antigen (Pearce and MacDonald, 2002). Similar findings have been reported for other helminths (Lawrence et al., 1994) (Artis et al., 1999). Considering that NES is a mixture of different products secreted/excreted by *N. brasiliensis* adult worms, it was necessary to rule out here whether some of these products were inducing the generation of allergen-specific Th1 immune responses. As expected, the anti-allergic effect of NES resulted to be completely independent on the generation of Th1 immune responses as demonstrated using IFN-γ-deficient mice. Moreover, sensitization with OVA+NES/alum was not able to stimulate the production of serum OVA-specific IgG2a in mice (which is induced by IFN-γ), a further finding arguing against the generation of NES-mediated OVA-specific Th1 responses.

A more reasonable explanation for the suppressive effect of NES on OVA-specific Th2 responses would be the generation of IL-10-producing Tr cells stimulated by this helminth product. This would agree with previous findings demonstrating that infection with *N. brasiliensis* inhibited the development of allergen-induced airway eosinophilia, an effect that was reported to be mediated by IL-10 (Wohlleben et al., 2004). Moreover, recent publications have lead to the hypothesis that infections of humans with helminths induce the production of IL-10, which interferes with Th2 cell effector mechanisms (Biggelaar et al., 2000) (Maizels et al., 2004). Therefore, to define the role of IL-10 in the protection induced by NES on the OVA-induced Th2 responses two complementary approaches were selected: first, IL-10-mediated signaling was blocked in mice during the entire OVA sensitization period by using a

monoclonal antibody directed against the IL-10R and second, IL-10-deficient mice were used. Unexpectedly, none of these approaches could demonstrate a role of IL-10 in the anti-allergic effect of NES. It suggests that living *N. brasiliensis* worms and *N. brasiliensis*-derived products seem to induce the suppression of OVA-induced Th2 responses through different mechanisms. However, the generation of immunregulation induced by NES cannot be ruled out considering that a variety of murine cells has been shown to display regulatory function *in vitro* or *in vivo* in an IL-10-independent manner. Besides the so-called T regulatory 1 cells (Tr1 cells) which mediate their suppressive effects mainly through the production of IL-10, $CD4^+CD25^+$ T cells, Th3 cells (producing mainly TGF-β) as well as subpopulations of $CD8^+$ T cells, TCR $\gamma\delta^+$ T cells and also NKT cells have been shown to exert suppressive functions *in vivo* (Battaglia et al., 2002) (Robinson et al., 2004).

It would be posible that the generation of $CD4^+CD25^+$ T regulatory cells were stimulated by NES, inducing the IL-10-independent suppression of OVA-induced Th2 responses. Indeed, the infection with another helminth, *S. mansoni*, induces the generation of $CD4^+CD25^+$ Tr cells in mice which can inhibit immune responses partially through a IL-10-independent mechanism (McKee and Pearce, 2004). Interestingly, some recent studies demonstrated that naturally arising $CD4^+CD25^+$ Tr cells in normal mice selectively express several members of the TLR family and that stimulation of these cells through TLRs can expand them and strengthen their suppressive activity (Caramalho et al., 2003). Although no common helminth molecular pattern or recognition receptor has been identified so far, NES might act through a still unknown receptor to elicit the proliferation or alternatively, prolong the survival of $CD4^+CD25^+$ Tr cells and augment their *in vitro* suppressive activity. Considering that $CD4^+CD25^+$ Tr cells activated by a specific antigen seem to exert antigen-nonspecific suppression, Tr cells stimulated by NES-derived antigens may also suppress OVA-induced Th2 immune responses in a bystander manner.

On the other hand, it is of note that the TGF-β-dependency of the anti-allergy effect of NES was not investigated in this study. Since homologues of TGF-β or TGF-β receptor family have been reported to be expressed by some helminth parasites (Davies et al., 1998) (Gomez-Escobar et al., 2000) and taking into account the immunosuppressive capacity of this cytokine (Wahl et al., 2004), the role of TGF-β on the ability of NES to suppress OVA-induced Th2 responses needs to be definitely clarified.

Moreover, other mechanisms inducing the generation of regulatory responses by helminths have been reported. An important advance in this matter has been the discovery of a new form of macrophages that arises in nematode infections, which operates through an IL-10-independent pathway (MacDonald et al., 1998). Nematode-elicited macrophages (NeMacs) have a different gene expression profile, are markedly cytostatic, preventing the proliferation of non-lymphoid cells, as well as T cells, and act through a contact-dependent mechanism that is independent of known inhibitors such as nitric oxide, prostaglandins or the cytokines IL-10 or TGF-β (Loke et al., 2000) (Loke et al., 2002) (Nair et al., 2003). First found in the peritoneum of mice infected with *Brugia malayi*, similar cells occur in animals that are exposed to other nematodes such as *N. brasiliensis* and their secreted products (Allen and MacDonald, 1998) (Smith et al., 2004). Of importance, NES was shown to induce NeMacs with capacity to reduce the proliferation of a Th2 cell clone *in vitro* (Allen and MacDonald, 1998). The biological significance of this *in vitro* finding has not still been placed on firm *in vivo* footing but allows to speculate that NeMacs may be induced during the intraperitoneal application of NES, which in turn, would suppress OVA-specific Th2 cell responses.

In an attempt to elucidate the components of NES that were mediating the suppression of OVA-specific Th2 responses, this product was digested with proteinase K and then, the

activity of the protein-free fraction was evaluated in mice. Protein as well as non-protein fraction of NES were shown to have suppressive activity on OVA-specific Th2 responses. In addition, the use of heat-treated NES demonstrated that this suppressive activity was heat-resistant suggesting that both heat-resistant proteins and non-protein components of NES are mediating this effect. Some progress has been made in elucidating the NES proteins with immunosuppressive capacity. It was reported that nippocystatin, a NES-derived cysteine protease inhibitor (NbCys) is able to suppress OVA-specific proliferation and cytokine production of murine splenocytes (Dainichi et al., 2001). In addition, NbCys was also able to downregulate OVA-specific IgE (but not IgG1) levels in OVA/alum-treated mice. Although the heat-resistance of NbCys was not evaluated in that study, these data clearly demonstrated that protein components of NES could downmodulate immune responses to non-related antigens *in vivo*. Furthermore, among the non-protein candidates for the anti-allergy activity of NES, a lysophosphatidylserine family of molecules (lyso-PS) with acyl chains should be considered. This lipid species not found in mammalians were reported to be present in *S. mansoni* adult worms (Kleij et al., 2002). They were shown to be able to activate DC through TLR-2 inducing Tr cells. Alternatively, NES might contain lipoconjugates with potent ability to suppress Th2 allergic responses. The ability of lipoconjugates to influence the development of Th2 responses were already demonstrated using the synthetic lipopeptide LP40 (Akdis et al., 2003). Triggering of TLR-2 by LP40 was shown to inhibit allergen-induced IgE production and lung eosinophilic inflammation in a murine model of allergic asthma. A more detailed analysis of the different components of NES remains to be performed in order to identify specifically which NES-derivative constituents have suppressive activity on allergen-induced Th2 responses.

A interesting finding of the present study was the ability of NES to reduce OVA-specific Th2 responses in the presence of a strong NES-specific Th2 environment. When applied with

OVA/alum, NES suppressed OVA-induced airway eosinophilia and OVA-specific IgG1 and IgE serum levels but stimulated in parallel high levels of NES-specific IgG1 and IgE and could potentially induce airway eosinophilia (by applying NES intranasally). In agreement with this finding are studies in humans which suggest an association of helminth infections with generalized immune suppression in spite of the generation of helminth-specific Th2 responses (Gallin et al., 1988) (King et al., 1993) (Grogan et al., 1998). This limited degree of diminished responsiveness may represent a mechanism of the parasite to establish harmonious host-parasite interplay that allows long-term infections. Maintaining controlled Th2-cell reactivity through a homeostatic immunsuppressive mechanism would be also beneficial for the host inasmuch as potential pathogenic damage caused by unrestricted Th2-cell-mediated inflammation is prevented. Further studies are needed in order to elucidate the components of NES with Th2-inducing capacity. Studies performed by others suggest that although NES consists of several dozen components, only large-molecular weight glycoproteins actively generates Th2 responses (Balic et al., 2004) (Tawill et al., 2004) (Vijver et al., 2004). Among the glycoproteins already identified in NES are enzymes with acetylcholinesterase and platelet-activating factor acetylhydrolase activities which should be evaluated as potential Th2-promoting components (Grigg et al., 1996).

Taken together, these results demonstrate that NES could be used as a potential adjuvant to prevent the development of allergic Th2 responses through a still unknown mechanism independent upon IFN-γ and IL-10. The ability of NES to suppress allergen-mediated Th2 responses in the presence of strong NES-specific Th2 environment confirm previous reports in humans describing the capacity of helminth infections to downregulate host immunity inducing simultaneously the generation of helminth-specific Th2 responses (Gallin et al., 1988) (King et al., 1993) (Grogan et al., 1998). Heat-resistant proteins as well as non-protein

components of NES seems to be the molecules responsible for its suppressive activity on allergen-induced Th2 responses.

In conclusion, the results of my thesis suggest that microorganism-derived vaccines are promising candidates to prevent the development of allergic diseases. My data show that all tested Th1-responses inducing adjuvants (live BCG, hk-BCG, CpG and PPD) have suppressive effects on the development of allergen-induced Th2 responses, which were to a great extent associated with the presence of IFN-γ and IL-12. This evidence strengthens the idea that Th1 cells may be suppressive, and that immune deviation from Th2 to a Th1 response may be a legitimate strategy to prevent allergic diseases. On the other hand, my results demonstrated that helminth-derived components such as NES are able to suppress allergen-induced Th2 responses in an IFN-γ- and IL-10-independent manner suggesting that other undefined immunregulatory connections are playing a role in the control of Th2-mediated pathologic responses. Although my results using DC as carriers for anti-allergy vaccines were not successful, dendritic cells may be essential to induce regulatory responses. Novel DC-based strategies to effectively prevent the generation of allergic responses will certainly be designed in the near future. Future investigations will allow to integrate these varying regulatory networks, thereby devising means of adapting these strategies for *in vivo* use to prevent human allergic diseases.

6. REFERENCES

Aaby, P., Shaheen, S. O., Heyes, C. B., Goudiaby, A., Hall, A. J., Shiell, A. W., Jensen, H., and Marchant, A. (2000). Early BCG vaccination and reduction in atopy in Guinea-Bissau. Clin Exp Allergy *30*, 644-650.

Aberg, N., Hesselmar, B., Aberg, B., and Eriksson, B. (1995). Increase of asthma, allergic rhinitis and eczema in Swedish schoolchildren between 1979 and 1991. Clin Exp Allergy *25*, 815-819.

Akdis, C. A., Kussebi, F., Pulendran, B., Akdis, M., Lauener, R. P., Schmidt-Weber, C. B., Klunker, S., Isitmangil, G., Hansjee, N., Wynn, T. A., *et al.* (2003). Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. Eur J Immunol *33*, 2717-2726.

Akdis, M., Verhagen, J., Taylor, A., Karamloo, F., Karagiannidis, C., Crameri, R., Thunberg, S., Deniz, G., Valenta, R., Fiebig, H., *et al.* (2004). Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med *199*, 1567-1575.

Akinbami, L. J., and Schoendorf, K. C. (2002). Trends in childhood asthma: prevalence, health care utilization, and mortality. Pediatrics *110*, 315-322.

Allen, J. E., and MacDonald, A. S. (1998). Profound suppression of cellular proliferation mediated by the secretions of nematodes. Parasite Immunol *20*, 241-247.

Alm, J. S., Lilja, G., Pershagen, G., and Scheynius, A. (1997). Early BCG vaccination and development of atopy. Lancet *350*, 400-403.

Anderson, H. R., Poloniecki, J. D., Strachan, D. P., Beasley, R., Bjorksten, B., Asher, M. I., and Group, I. P. S. (2001). Immunization and symptoms of atopic disease in children: results from the International Study of Asthma and Allergies in Childhood. Am J Public Health *91*, 1126-1129.

Araujo, M. I., Lopes, A. A., Medeiros, M., Cruz, A. A., Sousa-Atta, L., Sole, D., and Carvalho, E. M. (2000). Inverse association between skin response to aeroallergens and *Schistosoma mansoni* infection. Int Arch Allergy Immunol *123*, 145-148.

Artis, D., Potten, C. S., Else, K. J., Finkelman, F. D., and Grencis, R. K. (1999). *Trichuris muris*: host intestinal epithelial cell hyperproliferation during chronic infection is regulated by interferon-gamma. Exp Parasitol *92*, 144-153.

Asher, M. I., and ISAAC Steeriing Committee. (1998). Worldwide variations in the prevalence of asthma symptoms: the International Study of Asthma and Allergies in Childhood (ISAAC). Eur Respir J *12*, 315-335.

Bach, J. F. (2001). Protective role of infections and vaccinations on autoimmune diseases. J Autoimmun *16*, 347-353.

Bager, P., Rostgaard, K., Nielsen, N. M., Melbye, M., and Westergaard, T. (2003). Age at bacille Calmette-Guerin vaccination and risk of allergy and asthma. Clin Exp Allergy *33*, 1512-1517.

Balic, A., Harcus, Y., Holland, M. J., and Maizels, R. M. (2004). Selective maturation of dendritic cells by *Nippostrongylus brasiliensis*-secreted proteins drives Th2 immune responses. Eur J Immunol *34*, 3047-3059.

Banerjee, B., Kelly, K. J., Fink, J. N., Henderson, J. D. J., Bansal, N. K., and Kurup, V. P. (2004). Modulation of airway inflammation by immunostimulatory CpG oligodeoxynucleotides in a murine model of allergic aspergillosis. Infect Immun *72*, 6087-6094.

Bashir, M. E., Andersen, P., Fuss, I. J., Shi, H. N., and Nagler-Anderson, C. (2002). An enteric helminth infection protects against an allergic response to dietary antigen. J Immunol *169*, 3284-3292.

Battaglia, M., Blazar, B. R., and Roncarolo, M. G. (2002). The puzzling world of murine T regulatory cells. Microbes Infect *4*, 559-566.

Berberich, C., Ramirez-Pineda, J. R., Hambrecht, C., Alber, G., Skeiky, Y. A., and Moll, H. (2003). Dendritic cell (DC)-based protection against an intracellular pathogen is dependent upon DC-derived IL-12 and can be induced by molecularly defined antigens. J Immunol *170*, 3171-3179.

Berger, T. G., and Schultz, E. S. (2003). Dendritic cell-based immunotherapy. Curr Top Microbiol Immunol *276*, 163-197.

Biggelaar, A. H. v. d., Ree, R. v., Rodrigues, L. C., Lell, B., Deelder, A. M., Kremsner, P. G., and Yazdanbakhsh, M. (2000). Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. Lancet *356*, 1723-1727.

Braun-Fahrlander, C., Riedler, J., Herz, U., Eder, W., Waser, M., Grize, L., Maisch, S., Carr, D., Gerlach, F., Bufe, A., *et al.* (2002). Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med *347*, 869-877.

Bullens, D. M., Keybus, C. V. D., Dilissen, E., Kasran, A., and Ceuppens, J. L. (2004). Allergen-specific T cells from birch-pollen-allergic patients and healthy controls differ in T helper 2 cytokine and in interleukin-10 production. Clin Exp Allergy *34*, 879-887.

Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M., and Demengeot, J. (2003). Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med *197*, 403-411.

Cavallo, G. P., Elia, M., Giordano, D., Baldi, C., and Cammarota, R. (2002). Decrease of specific and total IgE levels in allergic patients after BCG vaccination: preliminary report. Arch Otolaryngol Head Neck Surg *128*, 1058-1060.

Chiang, D. J., Ye, Y. L., Chen, W. L., Lee, Y. L., Hsu, N. Y., and Chiang, B. L. (2003). Ribavirin or CpG DNA sequence-modulated dendritic cells decrease the IgE level and airway inflammation. Am J Respir Crit Care Med *168*, 575-580.

Choi, I. S., and Koh, Y. I. (2002). Therapeutic effects of BCG vaccination in adult asthmatic patients: a randomized, controlled trial. Ann Allergy Asthma Immunol *88*, 584-591.

Cohn, L., Homer, R. J., Marinov, A., Rankin, J., and Bottomly, K. (1997). Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. J Exp Med *186*, 1737-1747.

Cottrez, F., Hurst, S. D., Coffman, R. L., and Groux, H. (2000). T regulatory cells 1 inhibit a Th2-specific response in vivo. J Immunol *165*, 4848-4853.

Dainichi, T., Maekawa, Y., Ishii, K., Zhang, T., Nashed, B. F., Sakai, T., Takashima, M., and Himeno, K. (2001). Nippocystatin, a cysteine protease inhibitor from *Nippostrongylus brasiliensis*, inhibits antigen processing and modulates antigen-specific immune response. Infect Immun *69*, 7380-7386.

Das, J., Chen, C. H., Yang, L., Cohn, L., Ray, P., and Ray, A. (2001). A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat Immunol *2*, 45-50.

David, T., Thomas, C., Zaccone, P., Dunne, D. W., and Cooke, A. (2004). The impact of infection on the incidence of autoimmune disease. Curr Top Med Chem *4*, 521-529.

Davies, S. J., Shoemaker, C. B., and Pearce, E. J. (1998). A divergent member of the transforming growth factor beta receptor family from *Schistosoma mansoni* is expressed on the parasite surface membrane. J Biol Chem *273*, 11234-11240.

Deurloo, D. T., Esch, B. C. v., Hofstra, C. L., Nijkamp, F. P., and Oosterhout, A. J. v. (2001). CTLA4-IgG reverses asthma manifestations in a mild but not in a more "severe" ongoing murine model. Am J Respir Cell Mol Biol *25*, 751-760.

Devenny, A., Wassall, H., Ninan, T., Omran, M., Khan, S. D., and Russell, G. (2004). Respiratory symptoms and atopy in children in Aberdeen: questionnaire studies of a defined school population repeated over 35 years. BMJ *329*, 489-490.

Devereux, G., Ayatollahi, T., Ward, R., Bromly, C., Bourke, S. J., Stenton, S. C., and Hendrick, D. J. (1996). Asthma, airways responsiveness and air pollution in two contrasting districts of northern England. Thorax *51*, 169-174.

Ebner, C., Schenk, S., Najafian, N., Siemann, U., Steiner, R., Fischer, G. W., Hoffmann, K., Szepfalusi, Z., Scheiner, O., and Kraft, D. (1995). Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. J Immunol *154*, 1932-1940.

Eder, W., and Mutius, E. v. (2004). Hygiene hypothesis and endotoxin: what is the evidence? Curr Opin Allergy Clin Immunol *4*, 113-117.

Elias, J. A., Zhu, Z., Chupp, G., and Homer, R. J. (1999). Airway remodeling in asthma. J Clin Invest *104*, 1001-1006.

Erb, K. J., Holloway, J. W., Sobeck, A., Moll, H., and Gros, G. L. (1998). Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. J Exp Med *187*, 561-569.

Erb, K. J., and Wohlleben, G. (2002). Novel vaccines protecting against the development of allergic disorders: a double-edged sword? Curr Opin Immunol *14*, 633-643.

Ernst, J. D. (1998). Macrophage receptors for *Mycobacterium tuberculosis*. Infect Immun *66*, 1277-1281.

Finotto, S., and Glimcher, L. (2004). T cell directives for transcriptional regulation in asthma. Springer Semin Immun *25*, 281-294.

Gajewski, T. F., and Fitch, F. W. (1988). Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J Immunol *140*, 4245-4252.

Gallin, M., Edmonds, K., Ellner, J. J., Erttmann, K. D., White, A. T., Newland, H. S., Taylor, H. R., and Greene, B. M. (1988). Cell-mediated immune responses in human infection with *Onchocerca volvulus*. J Immunol *140*, 1999-2007.

Gavett, S. H., Chen, X., Finkelman, F., and Wills-Karp, M. (1994). Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. Am J Respir Cell Mol Biol *10*, 587-593.

Geijtenbeek, T. B., Vliet, S. J. V., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B., and Kooyk, Y. V. (2003). Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med *197*, 7-17.

Gomez-Escobar, N., Gregory, W. F., and Maizels, R. M. (2000). Identification of tgh-2, a filarial nematode homolog of *Caenorhabditis elegans* daf-7 and human transforming growth factor beta, expressed in microfilarial and adult stages of *Brugia malayi*. Infect Immun *68*, 6402-6410.

Gonzalo, J. A., Lloyd, C. M., Kremer, L., Finger, E., Martinez-A, C., Siegelman, M. H., Cybulsky, M., and Gutierrez-Ramos, J. C. (1996). Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. J Clin Invest *98*, 2332-2345.

Graffi, S. J., Dekan, G., Stingl, G., and Epstein, M. M. (2002). Systemic administration of antigen-pulsed dendritic cells induces experimental allergic asthma in mice upon aerosol antigen rechallenge. Clin Immunol *103*, 176-184.

Grigg, M. E., Gounaris, K., and Selkirk, M. E. (1996). Characterization of a platelet-activating factor acetylhydrolase secreted by the nematode parasite *Nippostrongylus brasiliensis*. Biochem J *317*, 541-547.

Grogan, J. L., Kremsner, P. G., Deelder, A. M., and Yazdanbakhsh, M. (1998). Antigen-specific proliferation and interferon-gamma and interleukin-5 production are down-regulated during *Schistosoma haematobium* infection. J Infect Dis *177*, 1433-1437.

Grunewald, S. M., Hahn, C., Wohlleben, G., Teufel, M., Major, T., Moll, H., Brocker, E. B., and Erb, K. J. (2002). Infection with influenza a virus leads to flu antigen-induced cutaneous anaphylaxis in mice. J Invest Dermatol *118*, 645-651.

Gupta, R., Sheikh, A., Strachan, D. P., and Anderson, H. R. (2004). Burden of allergic disease in the UK: secondary analyses of national databases. Clin Exp Allergy *34*, 520-526.

Gutierrez-Ramos, J. C., Lloyd, C., Kapsenberg, M. L., Gonzalo, J. A., and Coyle, A. J. (2000). Non-redundant functional groups of chemokines operate in a coordinate manner during the inflammatory response in the lung. Immunol Rev *177*, 31-42.

Hamelmann, E., and Gelfand, E. W. (1999). Role of IL-5 in the development of allergen-induced airway hyperresponsiveness. Int Arch Allergy Immunol *120*, 8-16.

Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G. L., Irvin, C. G., and Gelfand, E. W. (1997). Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am J Respir Crit Care Med *156*, 766-775.

Hammad, H., Heer, H. J. d., Soullie, T., Angeli, V., Trottein, F., Hoogsteden, H. C., and Lambrecht, B. N. (2004). Activation of peroxisome proliferator-activated receptor-gamma in dendritic cells inhibits the development of eosinophilic airway inflammation in a mouse model of asthma. Am J Pathol *164*, 263-271.

Hansen, G., Berry, G., DeKruyff, R. H., and Umetsu, D. T. (1999). Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. J Clin Invest *103*, 175-183.

Hansen, G., Yeung, V. P., Berry, G., Umetsu, D. T., and DeKruyff, R. H. (2000). Vaccination with heat-killed Listeria as adjuvant reverses established allergen-induced airway hyperreactivity and inflammation: role of CD8+ T cells and IL-18. J Immunol *164*, 223-230.

Hartert, T. V., and Peebles, R. S. Jr. (2000). Epidemiology of asthma: the year in review. Curr Opin Pulm Med *6*, 4-9.

Hattori, H., Okano, M., Yamamoto, T., Yoshino, T., Yamashita, Y., Watanabe, T., Satoskar, A. R., Harn, D. A., and Nishizaki, K. (2002). Intranasal application of purified protein derivative suppresses the initiation but not the exacerbation of allergic rhinitis in mice. Clin Exp Allergy *32*, 951-959.

Hauber, H. P., Bergeron, C., and Hamid, Q. (2004). IL-9 in allergic inflammation. Int Arch Allergy Immunol *134*, 79-87.

Healer, J., Ashall, F., and Maizels, R. M. (1991). Characterization of proteolytic enzymes from larval and adult *Nippostrongylus brasiliensis*. Parasitology *103*, 305-314.

Heer, H. J. d., Hammad, H., Soullie, T., Hijdra, D., Vos, N., Willart, M. A., Hoogsteden, H. C., and Lambrecht, B. N. (2004). Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. J Exp Med *200*, 89-98.

Heikenwalder, M., Polymenidou, M., Junt, T., Sigurdson, C., Wagner, H., Akira, S., Zinkernagel, R., and Aguzzi, A. (2004). Lymphoid follicle destruction and

immunosuppression after repeated CpG oligodeoxynucleotide administration. Nat Med *10*, 187-192.

Heldwein, K. A., Liang, M. D., Andresen, T. K., Thomas, K. E., Marty, A. M., Cuesta, N., Vogel, S. N., and Fenton, M. J. (2003). TLR2 and TLR4 serve distinct roles in the host immune response against *Mycobacterium bovis* BCG. J Leukoc Biol *74*, 277-286.

Hertzen, L. C. v., and Haahtela, T. (2004). Asthma and atopy - the price of affluence? Allergy *59*, 124-137.

Hertzen, L. v., Klaukka, T., Mattila, H., and Haahtela, T. (1999). *Mycobacterium tuberculosis* infection and the subsequent development of asthma and allergic conditions. J Allergy Clin Immunol *104*, 1211-1214.

Hilkens, C. M., Kalinski, P., Boer, M. d., and Kapsenberg, M. L. (1997). Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. Blood *90*, 1920-1926.

Hirsch, T., Weiland, S. K., Mutius, E. v., Safeca, A. F., Grafe, H., Csaplovics, E., Duhme, H., Keil, U., and Leupold, W. (1999). Inner city air pollution and respiratory health and atopy in children. Eur Respir J *14*, 669-677.

Hoffjan, S., and Ober, C. (2002). Present status on the genetic studies of asthma. Curr Opin Immunol *14*, 709-717.

Holland, M. J., Harcus, Y. M., Balic, A., and Maizels, R. M. (2005). Th2 induction by *Nippostrongylus* secreted antigens in mice deficient in B cells, eosinophils or MHC Class I-related receptors. Immunol Lett *96*, 93-101.

Holland, M. J., Harcus, Y. M., Riches, P. L., and Maizels, R. M. (2000). Proteins secreted by the parasitic nematode *Nippostrongylus brasiliensis* act as adjuvants for Th2 responses. Eur J Immunol *30*, 1977-1987.

Hopfenspirger, M. T., and Agrawal, D. K. (2002). Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-presensitized mice. J Immunol *168*, 2516-2522.

Horner, A. A., Uden, J. H. V., Zubeldia, J. M., Broide, D., and Raz, E. (2001). DNA-based immunotherapeutics for the treatment of allergic diseases. Immunological Rev *179*, 102-118.

Hussein, A. S., Harel, M., and Selkirk, M. E. (2002). A distinct family of acetylcholinesterases is secreted by *Nippostrongylus brasiliensis*. Mol Biochem Parasitol *123*, 125-134.

Hylkema, M. N., Timens, W., Luinge, M., Werf, N. V. D., and Hoekstra, M. O. (2002). The effect of bacillus Calmette-Guerin immunization depends on the genetic predisposition to Th2-type responsiveness. Am J Respir Cell Mol Biol *27*, 244-249.

Janeway, C. A., Travers, P., Walport, M., and Shlomchik, M. (2001). Immunobiology: the immune system in health and disease, Fifth Edition edn (New York, Garland Publishing Inc).

Jang, A. S., and Son, M. H. (2002). The association of airway hyperresponsiveness and tuberculin responses. Allergy *57*, 341-345.

Jentoft, H. F., Omenaas, E., Eide, G. E., and Gulsvik, A. (2002). Absence of relationship between tuberculin reactivity and asthmatic symptoms, level of FEV1 and bronchial hyperresponsiveness in BCG vaccinated young adults. Allergy *57*, 336-340.

Kawakami, T., and Galli, S. J. (2002). Regulation of mast-cell and basophil function and survival by IgE. Nat Rev Immunol *2*, 773-786.

King, C. L., Mahanty, S., Kumaraswami, V., Abrams, J. S., Regunathan, J., Jayaraman, K., Ottesen, E. A., and Nutman, T. B. (1993). Cytokine control of parasite-specific anergy in human lymphatic filariasis. Preferential induction of a regulatory T helper type 2 lymphocyte subset. J Clin Invest *92*, 1667-1673.

Kita, H., Kaneko, M., Bartemes, K. R., Weiler, D. A., Schimming, A. W., Reed, C. E., and Gleich, G. J. (1999). Does IgE bind to and activate eosinophils from patients with allergy? J Immunol *162*, 6901-6911.

Kleij, D. v. d., Latz, E., Brouwers, J. F., Kruize, Y. C., Schmitz, M., Kurt-Jones, E. A., Espevik, T., Jong, E. C. d., Kapsenberg, M. L., Golenbock, D. T*., et al.* (2002). A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. J Biol Chem *277*, 48122-48129.

Kline, J. N., Krieg, A. M., Waldschmidt, T. J., Ballas, Z. K., Jain, V., and Businga, T. R. (1999). CpG oligodeoxynucleotides do not require TH1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. J Allergy Clin Immunol *104*, 1258-1264.

Kline, J. N., Waldschmidt, T. J., Businga, T. R., Lemish, J. E., Weinstock, J. V., Thorne, P. S., and Krieg, A. M. (1998). Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. J Immunol *160*, 2555-2559.

Koh, Y. I., Choi, I. S., and Kim, W. Y. (2001). BCG infection in allergen-presensitized rats suppresses Th2 immune response and prevents the development of allergic asthmatic reaction. J Clin Immunol *21*, 51-59.

Kondo, M., Tamaoki, J., Takeyama, K., Nakata, J., and Nagai, A. (2002). Interleukin-13 induces goblet cell differentiation in primary cell culture from Guinea pig tracheal epithelium. Am J Respir Cell Mol Biol *27*, 536-541.

Krieg, A. M. (2002). CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol *20*, 709-760.

Kroegel, C., Julius, P., Matthys, H., Virchow, J. C. J., and Luttmann, W. (1996). Endobronchial secretion of interleukin-13 following local allergen challenge in atopic asthma: relationship to interleukin-4 and eosinophil counts. Eur Respir J *9*, 899-904.

Kuipers, H., Heirman, C., Hijdra, D., Muskens, F., Willart, M., Meirvenne, S. v., Thielemans, K., Hoogsteden, H. C., and Lambrecht, B. N. (2004). Dendritic cells retrovirally overexpressing IL-12 induce strong Th1 responses to inhaled antigen in the lung but fail to revert established Th2 sensitization. J Leukoc Biol *76*, 1028-1038.

Kuipers, H., Hijdra, D., Vries, V. C. D., Hammad, H., Prins, J. B., Coyle, A. J., Hoogsteden, H. C., and Lambrecht, B. N. (2003). Lipopolysaccharide-induced suppression of airway Th2 responses does not require IL-12 production by dendritic cells. J Immunol *171*, 3645-3654.

Laan, L. J. v. d., Dopp, E. A., Haworth, R., Pikkarainen, T., Kangas, M., Elomaa, O., Dijkstra, C. D., Gordon, S., Tryggvason, K., and Kraal, G. (1999). Regulation and functional involvement of macrophage scavenger receptor MARCO in clearance of bacteria in vivo. J Immunol *162*, 939-947.

Lack, G., Bradley, K. L., Hamelmann, E., Renz, H., Loader, J., Leung, D. Y., Larsen, G., and Gelfand, E. W. (1996). Nebulized IFN-gamma inhibits the development of secondary allergic responses in mice. J Immunol *157*, 1432-1439.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lambrecht, B. N., and Hammad, H. (2003a). The other cells in asthma: dendritic cell and epithelial cell crosstalk. Curr Opin Pulm Med *9*, 34-41.

Lambrecht, B. N., and Hammad, H. (2003b). Taking our breath away: dendritic cells in the pathogenesis of asthma. Nat Rev Immunol *3*, 994-1003.

Lambrecht, B. N., Salomon, B., Klatzmann, D., and Pauwels, R. A. (1998). Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. J Immunol *160*, 4090-4097.

Lambrecht, B. N., Veerman, M. D., Coyle, A. J., Gutierrez-Ramos, J. C., Thielemans, K., and Pauwels, R. A. (2000). Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. J Clin Invest *106*, 551-559.

Laoukili, J., Perret, E., Willems, T., Minty, A., Parthoens, E., Houcine, O., Coste, A., Jorissen, M., Marano, F., Caput, D., and Tournier, F. (2001). IL-13 alters mucociliary differentiation and ciliary beating of human respiratory epithelial cells. J Clin Invest *108*, 1817-1824.

Lawrence, R. A., Allen, J. E., Osborne, J., and Maizels, R. M. (1994). Adult and microfilarial stages of the filarial parasite *Brugia malayi* stimulate contrasting cytokine and Ig isotype responses in BALB/c mice. J Immunol *153*, 1216-1224.

Lawrence, R.A., Gray, C.A., Osborne, J., and Maizels, R.M. (1996). *Nippostrongylus brasiliensis*: cytokine responses and nematode expulsion in normal and IL-4-deficient mice. Exp Parasitol *84*, 65-73.

Lewkowich, I. P., and HayGlass, K. T. (2002). Endogenous IFN-gamma and IL-18 production directly limit induction of type 2 immunity in vivo. Eur J Immunol *32*, 3536-3545.

Li, X. M., Chopra, R. K., Chou, T. Y., Schofield, B. H., Wills-Karp, M., and Huang, S. K. (1996). Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice. J Immunol *157*, 3216-3219.

Lima, C., Perini, A., Garcia, M. L., Martins, M. A., Teixeira, M. M., and Macedo, M. S. (2002). Eosinophilic inflammation and airway hyper-responsiveness are profoundly inhibited by a helminth (*Ascaris suum*) extract in a murine model of asthma. Clin Exp Allergy *32*, 1659-1666.

Loke, P., MacDonald, A. S., Robb, A., Maizels, R. M., and Allen, J. E. (2000). Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. Eur J Immunol *30*, 2669-2678.

Loke, P., Nair, M. G., Parkinson, J., Guiliano, D., Blaxter, M., and Allen, J. E. (2002). IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. BMC Immunol *3*, 7.

Lucas, S., Ghilardi, N., Li, J., and Sauvage, F. J. d. (2003). IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. Proc Natl Acad Sci USA *100*, 15047-15052.

Lutz, M. B., Kukutsch, N., Ogilvie, A. L. J., Rößner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Meth *223*, 77-92.

Lynch, N. R., Hagel, I., Perez, M., Prisco, M. C. D., Lopez, R., and Alvarez, N. (1993). Effect of anthelmintic treatment on the allergic reactivity of children in a tropical slum. J Allergy Clin Immunol *92*, 404-411.

MacDonald, A. S., Maizels, R. M., Lawrence, R. A., Dransfield, I., and Allen, J. E. (1998). Requirement for in vivo production of IL-4, but not IL-10, in the induction of proliferative suppression by filarial parasites. J Immunol *160*, 4124-4132.

Maizels, R. M., Balic, A., Gomez-Escobar, N., Nair, M., Taylor, M. D., and Allen, J. E. (2004). Helminth parasites: masters of regulation. Immunol Rev *201*, 89-116.

Maizels, R. M., and Yazdanbakhsh, M. (2003). Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol *3*, 733-744.

Major, T., Wohlleben, G., Reibetanz, B., and Erb, K. J. (2002). Application of heat killed *Mycobacterium bovis*-BCG into the lung inhibits the development of allergen-induced Th2 responses. Vaccine *20*, 1532-1540.

Matricardi, P. M., Rosmini, F., Panetta, V., Ferrigno, L., and Bonini, S. (2002). Hay fever and asthma in relation to markers of infection in the United States. J Allergy Clin Immunol *110*, 381-387.

Matricardi, P. M., Rosmini, F., Riondino, S., Fortini, M., Ferrigno, L., Rapicetta, M., and Bonini, S. (2000). Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. BMJ *320*, 412-417.

McGeady, S. J. (2004). Immunocompetence and allergy. Pediatrics *113*, 1107-1113.

McKee, A. S., and Pearce, E. J. (2004). CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. J Immunol *173*, 1224-1231.

Mehta-Damani, A., Markowicz, S., and Engleman, E. G. (1995). Generation of antigen-specific CD4+ T cell lines from naive precursors. Eur J Immunol *25*, 1206-1211.

Mizuki, D., Miura, T., Sasaki, S., Mizuki, M., Madarame, H., and Nakane, A. (2001). Interference between host resistance to *Listeria monocytogenes* infection and ovalbumin-induced allergic responses in mice. Infect Immun *69*, 1883-1888.

Mochizuki, M., Bartels, J., Mallet, A. I., Christophers, E., and Schroder, J. M. (1998). IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. J Immunol *160*, 60-68.

Morokata, T., Ishikawa, J., Ida, K., and Yamada, T. (1999). C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses. Immunology *98*, 345-351.

Mosmann, T. R., Cherwinski, H., Bond, M., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine ctivities and secreted proteins. J Immunol *136*, 2348-2357.

Mutius, E. v., Pearce, N., Beasley, R., Cheng, S., Ehrenstein, O. v., Bjorksten, B., and Weiland, S. (2000). International patterns of tuberculosis and the prevalence of symptoms of asthma, rhinitis, and eczema. Thorax *55*, 449-453.

Nahori, M. A., Lagranderie, M., Lefort, J., Thouron, F., Joseph, D., Winter, N., Gicquel, B., Silva, J. R. L. e., and Vargaftig, B. B. (2001). Effects of *Mycobacterium bovis* BCG on the development of allergic inflammation and bronchial hyperresponsiveness in hyper-IgE BP2 mice vaccinated as newborns. Vaccine *19*, 1484-1495.

Nair, M. G., Cochrane, D. W., and Allen, J. E. (2003). Macrophages in chronic type 2 inflammation have a novel phenotype characterized by the abundant expression of Ym1 and Fizz1 that can be partly replicated in vitro. Immunol Lett *85*, 173-180.

Nicolle, D., Fremond, C., Pichon, X., Bouchot, A., Maillet, I., Ryffel, B., and Quesniaux, V. J. (2004a). Long-term control of *Mycobacterium bovis* BCG infection in the absence of Toll-like receptors (TLRs): investigation of TLR2-, TLR6-, or TLR2-TLR4-deficient mice. Infect Immun *72*, 6994-7004.

Nicolle, D. M., Pichon, X., Bouchot, A., Maillet, I., Erard, F., Akira, S., Ryffel, B., and Quesniaux, V. F. (2004b). Chronic pneumonia despite adaptive immune response to *Mycobacterium bovis* BCG in MyD88-deficient mice. Lab Invest *84*, 1305-1321.

Nyan, O. A., Walraven, G. E., Banya, W. A., Milligan, P., Sande, M. V. D., Ceesay, S. M., Prete, G. D., and McAdam, K. P. (2001). Atopy, intestinal helminth infection and total serum IgE in rural and urban adult Gambian communities. Clin Exp Allergy *31*, 1672-1678.

Omenaas, E., Jentoft, H. F., Vollmer, W. M., Buist, A. S., and Gulsvik, A. (2000). Absence of relationship between tuberculin reactivity and atopy in BCG vaccinated young adults. Thorax *55*, 454-458.

Parronchi, P., Carli, M. D., Manetti, R., Simonelli, C., Sampognaro, S., Piccinni, M. P., Macchia, D., Maggi, E., Prete, G. D., and Romagnani, S. (1992). IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. J Immunol *149*, 2977-2983.

Patel, K. D. (1998). Eosinophil tethering to interleukin-4-activated endothelial cells requires both P-selectin and vascular cell adhesion molecule-1. Blood *92*, 3904-3911.

Paul, W. E. (1997). Fundamental Immunology, Fourth Edition edn (Bethesda, MD, Lippincott Williams & Wilkins).

Pearce, E. J., and MacDonald, A. S. (2002). The immunobiology of schistosomiasis. Nat Rev Immunol *2*, 499-511.

Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E*., et al.* (2002). IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity *16*, 755-758.

Ramirez-Pineda, J. R., Frohlich, A., Berberich, C., and Moll, H. (2004). Dendritic cells (DC) activated by CpG DNA ex vivo are potent inducers of host resistance to an intracellular pathogen that is independent of IL-12 derived from the immunizing DC. J Immunol *172*, 6281-6289.

Randolph, D. A., Stephens, R., Carruthers, C. J. L., and Chaplin, D. D. (1999). Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. J Clin Invest *104*, 1021-1029.

Rautava, S., Kalliomaki, M., and Isolauri, E. (2002). Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. J Allergy Clin Immunol *109*, 119-121.

Rha, Y. H., Taube, C., Haczku, A., Joetham, A., Takeda, K., Duez, C., Siegel, M., Aydintug, M. K., Born, W. K., Dakhama, A., and Gelfand, E. W. (2002). Effect of microbial heat shock proteins on airway inflammation and hyperresponsiveness. J Immunol *169*, 5300-5307.

Riffo-Vazquez, Y., Spina, D., Page, C., Tormay, P., Singh, M., Henderson, B., Coates, A. (2004). Effect of *Mycobacterium tuberculosis* chaperonins on bronchial eosinophilia and hyper-responsiveness in a murine model of allergic inflammation. Clin Exp Immunol *34*, 712-719.

Robinson, D. S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A. M., Corrigan, C., Durham, S. R., and Kay, A. B. (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med *326*, Jan 30.

Robinson, D. S., Larche, M., and Durham, S. R. (2004). Tregs and allergic disease. J Clin Invest *114*, 1389-1397.

Rodrigo, G. J., Rodrigo, C., and Hall, J. B. (2004). Acute asthma in adults: a review. Chest *125*, 1081-1102.

Romagnani, S. (1992). Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. Int Arch Allergy Immunol *98*, 279-285.

Rook, G. A., and Brunet, L. R. (2002). Give us this day our daily germs. Biologist (London) *49*, 145-149.

Saito, A., Okazaki, H., Sugawara, I., Yamamoto, K., and Takizawa, H. (2003). Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. Int Arch Allergy Immunol *132*, 168-176.

Sayers, I., Severn, W., Scanga, C. B., Hudson, J., G, G. L. G., and Harper, J. L. (2004). Suppression of allergic airway disease using mycobacterial lipoglycans. J Allergy Clin Immunol *114*, 302-309.

Schmitz, N., Kurrer, M., and Kopf, M. (2003). The IL-1 receptor 1 is critical for Th2 cell type airway immune responses in a mild but not in a more severe asthma model. Eur J Immunol *33*, 991-1000.

Schon-Hegrad, M. A., Oliver, J., McMenamin, P. G., and Holt, P. G. (1991). Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. J Exp Med *173*, 1345-1356.

Sequeira, J. F., Cesic, D., Keser, G., Bukelica, M., Karanagnostis, S., Khamashta, M. A., and Hughes, G. R. (1993). Allergic disorders in systemic lupus erythematosus. Lupus *2*, 187-191.

Serebrisky, D., Teper, A. A., Huang, C. K., Lee, S. Y., Zhang, T. F., Schofield, B. H., Kattan, M., Sampson, H. A., and Li, X. M. (2000). CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. J Immunol *165*, 5906-5912.

Seya, T., Matsumoto, M., Tsuji, S., Begum, N. A., Azuma, I., and Toyoshima, K. (2002). Structural-functional relationship of pathogen-associated molecular patterns: lessons from BCG cell wall skeleton and mycoplasma lipoprotein M161Ag. Microbes Infect *4*, 955-961.

Shaheen, S. O., Aaby, P., Hall, A. J., Barker, D. J., Heyes, C. B., Shiell, A. W., and Goudiaby, A. (1996). Measles and atopy in Guinea-Bissau. Lancet *347*, 1792-1796.

Sheikh, A., Smeeth, L., and Hubbard, R. (2003). There is no evidence of an inverse relationship between TH2-mediated atopy and TH1-mediated autoimmune disorders: Lack of support for the hygiene hypothesis. J Allergy Clin Immunol *111*, 131-135.

Shirakawa, T., Enomoto, T., Shimazu, S., and Hopkin, J. M. (1997). The inverse association between tuberculin responses and atopic disorder. Science *275*, 77-79.

Shirota, H., Sano, K., Kikuchi, T., Tamura, G., and Shirato, K. (2000). Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. J Immunol *164*, 5575-5582.

Skadhauge, L. R., Christensen, K., Kyvik, K. O., and Sigsgaard, T. (1999). Genetic and environmental influence on asthma: a population-based study of 11,688 Danish twin pairs. Eur Respir J *13*, 8-14.

Smith, P., Walsh, C. M., Mangan, N. E., Fallon, R. E., Sayers, J. R., McKenzie, A. N., and Fallon, P. G. (2004). *Schistosoma mansoni* worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages. J Immunol *173*, 1240-1248.

Sparwasser, T., Miethke, T., Lipford, G., Borschert, K., Hacker, H., Heeg, K., and Wagner, H. (1997). Bacterial DNA causes septic shock. Nature Reviews *386*, 336-337.

Stene, L. C., and Nafstad, P. (2001). Relation between occurrence of type 1 diabetes and asthma. Lancet *357*, 607-608.

Strachan, D., Sibbald, B., Weiland, S., Ait-Khaled, N., Anabwani, G., Anderson, H. R., Asher, M. I., Beasley, R., Bjorksten, B., Burr, M*., et al.* (1997). Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC). Pediatr Allergy Immunol *8*, 161-176.

Strachan, D. P. (1989). Hay fever, hygiene, and household size. BMJ *299*, 1259-1260.

Su, Y. C., Peng, H. J., Wang, S. R., Han, S. H., and Tsai, J. J. (2001). Effects of BCG on ovalbumin-induced bronchial hyperreactivity in a guinea pig asthma model. J Microbiol Immunol Infect *34*, 25-34.

Sun, Y.C.J., and Hong, W. C. (2004). Do neutrophils actively participate in airway inflammation and remodeling in asthma?. *Chin Med J* 177, 1739-1742.

Sung, S., Rose, C. E., and Fu, S. M. (2001). Intratracheal priming with ovalbumin- and ovalbumin 323-339 peptide-pulsed dendritic cells induces airway hyperresponsiveness, lung eosinophilia, goblet cell hyperplasia, and inflammation. J Immunol *166*, 1261-1271.

Takaoka, A., Tanaka, Y., Tsuji, T., Jinushi, T., Hoshino, A., Asakura, Y., Mita, Y., Watanabe, K., Nakaike, S., Togashi, Y*., et al.* (2001). A critical role for mouse CXC chemokine(s) in pulmonary neutrophilia during Th type 1-dependent airway inflammation. J Immunol *167*, 2349-2353.

Takeda, K., and Akira, S. (2005). Toll-like receptors in innate immunity. Int Immunol *17*, 1-14.

Takeda, K., Tsutsui, H., Yoshimoto, T., Adachi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K., and Akira, S. (1998). Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity *8*, 383-390.

Taube, C., Dakhama, A., and Gelfand, E. W. (2004). Insights into the pathogenesis of asthma utilizing murine models. Int Arch Allergy Immunol *135*, 173-186.

Tawill, S., Goff, L. L., Ali, F., Blaxter, M., and Allen, J. E. (2004). Both free-living and parasitic nematodes induce a characteristic Th2 response that is dependent on the presence of intact glycans. Infect Immun *72*, 398-407.

Tokunaga, T., Yano, O., Kuramoto, E., Kimura, Y., Yamamoto, T., Kataoka, T., Yamamoto, S. (1992). Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. Microbiol Immunol *36*, 55-66.

Trinchieri, G., Pflanz, S., and Kastelein, R. A. (2003). The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. Immunity *19*, 641-644.

Trujillo, C., and Erb, K. J. (2003). Inhibition of allergic disorders by infection with bacteria or the exposure to bacterial products. Int J Med Microbiol *293*, 123-131.

Tulic, M. K., Wale, J. L., Holt, P. G., and Sly, P. D. (2000). Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide. Am J Respir Cell Mol Biol *22*, 604-612.

Uehori, J., Matsumoto, M., Tsuji, S., Akazawa, T., Takeuchi, O., Akira, S., Kawata, T., Azuma, I., Toyoshima, K., and Seya, T. (2003). Simultaneous blocking of human Toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by *Mycobacterium bovis* bacillus Calmette-Guerin peptidoglycan. Infect Immun *71*, 4238-4249.

Upham, J. W., Holt, B. J., Baron-Hay, M. J., Yabuhara, A., Hales, B. J., Thomas, W. R., Loh, R. K., O'Keeffe, P. T., Palmer, L., and Souef, P. N. L. (1995). Inhalant allergen-specific T-cell reactivity is detectable in close to 100% of atopic and normal individuals: covert responses are unmasked by serum-free medium. Clin Exp Allergy *25*, 634-642.

Vermaelen, K. Y., Carro-Muino, I., Lambrecht, B. N., and Pauwels, R. A. (2001). Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. J Exp Med *193*, 51-60.

Vijver, K. K. v. d., Hokke, C. H., Remoortere, A. v., Jacobs, W., Deelder, A. M., and Marck, E. A. V. (2004). Glycans of *Schistosoma mansoni* and keyhole limpet haemocyanin induce hepatic granulomas in vivo. Int J Parasitol *34*, 951-961.

Wahl, S. M., Swisher, J., McCartney-Francis, N., and Chen, W. (2004). TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. J Leukoc Biol *76*, 15-24.

Waite, D. A., Eyles, E. F., Tonkin, S. L., and O'Donnell, T. V. (1980). Asthma prevalence in Tokelauan children in two environments. Clin Allergy *10*, 71-75.

Wang, C. C., Nolan, T. J., Schad, G. A., and Abraham, D. (2001). Infection of mice with the helminth *Strongyloides stercoralis* suppresses pulmonary allergic responses to ovalbumin. Clin Exp Allergy *31*, 495-503.

Weikert, L. F., Lopez, J. P., Abdolrasulnia, R., Chroneos, Z. C., and Shepherd, V. L. (2000). Surfactant protein A enhances mycobacterial killing by rat macrophages through a nitric oxide-dependent pathway. Am J Physiol Lung Cell Mol Physiol *279*, L216-223.

Wheeler, A. W., Marshall, J. S., and Ulrich, J. T. (2001). A Th1-inducing adjuvant, MPL, enhances antibody profiles in experimental animals suggesting it has the potential to improve the efficacy of allergy vaccines. Int Arch Allergy Immunol *126*, 135-139.

Wills-Karp, M., Santeliz, J., and Karp, C. L. (2001). The germless theory of allergic disease: revisiting the hygiene hypothesis. Nat Rev Immunol *1*, 69-75.

Wohlleben, G., and Erb, K. J. (2001). Atopic disorders: a vaccine around the corner? Trends Immunol *22*, 618-626.

Wohlleben, G., Muller, J., Tatsch, U., Hambrecht, C., Herz, U., Renz, H., Schmitt, E., Moll, H., and Erb, K. J. (2003). Influenza A virus infection inhibits the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. J Immunol *170*, 4601-4611.

Wohlleben, G., Trujillo, C., Muller, J., Ritze, Y., Grunewald, S., Tatsch, U., and Erb, K. J. (2004). Helminth infection modulates the development of allergen-induced airway inflammation. Int Immunol *16*, 585-596.

Wong, G. W., Hui, D. S., Tam, C. M., Chan, H. H., Fok, T. F., Chan-Yeung, M., and Lai, C. K. (2001). Asthma, atopy and tuberculin responses in Chinese schoolchildren in Hong Kong. Thorax *56*, 770-773.

Wynn, T. A. (2003). IL-13 effector functions. Annu Rev Immunol *21*, 425-456.

Yang, X., Fan, Y., Wang, S., Han, X., Yang, J., Bilenki, L., and Chen, L. (2002). Mycobacterial infection inhibits established allergic inflammatory responses via alteration of cytokine production and vascular cell adhesion molecule-1 expression. Immunology *105*, 336-343.

Yeung, V. P., Gieni, R. S., Umetsu, D. T., and DeKruyff, R. H. (1998). Heat-killed *Listeria monocytogenes* as an adjuvant converts established murine Th2-dominated immune responses into Th1-dominated responses. J Immunol *161*, 4146-4152.

Yilmaz, M., Bingol, G., Altintas, D., and Kendirli, S. G. (2000). Correlation between atopic diseases and tuberculin responses. Allergy *55*, 664-667.

Yssel, H., Abbal, C., Pene, J., and Bousquet, J. (1998). The role of IgE in asthma. Clin Exp Allergy *28*, 104-109.

Zheng, W., and Flavell, R. A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell *89*, 587-596.

Zuany-Amorim, C., Sawicka, E., Manlius, C., Moine, A. L., Brunet, L. R., Kemeny, D. M., Bowen, G., Rook, G., and Walker, C. (2002). Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. Nat Med *8*, 625-629.

7. SUMMARY

According to the hygiene hypothesis, the exposure to infectious agents in early childhood prevents the development of allergen-specific Th2 immune responses because it establishes Th1-based immunity or alternatively, induces the generation of T regulatory cells. Based on this theory, the present study pretended to identify promising microorganism-derived vaccine candidates against allergic asthma in the murine model.

In the first part of this work, the efficacy of four different known Th1-inducing adjuvants, i.e. live BCG, heat-killed BCG, CpG and PPD, as components of vaccines aimed at inhibiting allergic asthma was compared. All the adjuvants were effective in inhibiting the development of allergen-induced airway eosinophilia, mucus production, and with the exception of PPD also airway hyperreactivity (AHR), when they were applied together with OVA/alum. Suppression of airway eosinophilia was not observed in IFN-γ- or IL-12-deficient mice (hk-BCG, CpG-ODN and PPD). Interestingly, live BCG was still able to suppress allergen-induced Th2 responses in the absence of either IFN-γ or IL-12. The effect of live BCG was also independent on IL-10-, TLR-2-, TLR-4- or MyD88-mediated signaling. When mice vaccinated with the different adjuvants together with OVA/alum were subjected to a second period of OVA/alum immunization, only live and hk-BCG were able to efficiently suppress the development of airway inflammation. This effect could be adoptively transferred by $CD4^+$ T cells. Taken together our data suggest that live BCG>>hk-BCG>CpG>PPD are effective in suppressing allergen-induced Th2 responses.

Secondly, the evaluation of a dendritic cell-based vaccination strategy leading to the induction of allergen-specific Th1 cells to protect against the development of allergen-specific Th2 responses was performed. The application of OVA-pulsed BM-DC maturated with CpG was unable to reduce airway eosinophilia and inflammation in OVA-immunized mice. OVA-specific IgG1 or IgE serum levels were also not reduced. The experiments using LC pulsed

with OVA yielded similar results. However, the mice vaccinated with CpG/OVA pulsed BM-DC had greatly enhanced levels of OVA-specific IgG2a in the serum, suggesting the induction of allergen-specific Th1 responses *in vivo*. Thus, these data suggest that the vaccination of mice with OVA-pulsed BM-DC matured with CpG or OVA-pulsed LC did not result in a reduction of allergen-specific Th2 responses in a murine model of severe atopic asthma.

Lastly, NES, an excretory/secretory product derived from the helminth *Nippostrongylus brasiliensis* was evaluated as a new potential adjuvant to prevent the development of allergic responses. The application of NES together with OVA/alum greatly inhibited the development of airway eosinophilia, airway goblet cell metaplasia and mucus production and the development of airway hyperreactivity after metacholine challenge. Furthermore, OVA-specific IgG1 and IgE levels in the serum were also strongly reduced. NES preparations contained small amounts of endotoxin, which may explain these results. However, the suppressive effects of NES on the development of allergen-specific Th2 responses was independent upon IFN-γ or TLR-4 and still observed in mice treated with LPS-depleted NES. NES reduced OVA-induced Th2 responses also in a IL-10-independent manner. In addition, the digestion with proteinase K or the heat-treatment of NES did not abolish its ability to inhibit allergen-induced Th2 responses. Interestingly, NES suppress OVA-specific Th2 responses *in vivo* in the presence of a strong NES-specific Th2 environment. Taken together our results suggest that the helminth *N. brasiliensis* secretes substances which interfere with the development of allergic Th2 responses.

In summary, distinct substances derived from microorganisms or helminths which may be used as potential adjuvants to prevent the development of allergic Th2 responses were identified. These findings contribute to the design of efficient vaccines protecting humans from developing allergic asthma.

8. ZUSAMMENFASSUNG

Die „Hygiene Hypothese" postuliert, dass der Kontakt mit Infektionserregern in der frühen Kindheit die Entwicklung von Th2-abhängigen allergischen Immunreaktionen verhindern kann, indem dadurch entweder eine vorrangig Th1-gerichtete Immunität etabliert wird oder alternativ die Bildung von regulatorischen T Zellen induziert wird. Basierend auf dieser Theorie zielte die vorliegende Arbeit darauf ab, Produkte von Mikroorganismen oder Würmern als mögliche Komponenten von Impfstoffen gegen Allergien zu testen.

Im ersten Teil dieser Arbeit wurden lebende BCG, Hitze abgetötete BCG (hk-BCG), CpG und PPD, die alle als Th1 Adjuvantien bekannt sind, auf ihre Effektivität getestet, allergisches Asthma in der Maus zu unterdrücken. Alle Adjuvantien konnten die durch Allergie induzierte Lungeneosinophilie, die Schleimproduktion in der Lunge und mit Ausnahme von PPD, die Lungenüberempfindlichkeit (AHR) unterdrücken, wenn sie zusammen mit OVA/alum verabreicht wurden. Die Lungeneosinophilie konnte jedoch nicht in IL-12 oder IFN-γ defizienten Mäusen durch die Applikation von hk-BCG, CpG oder PPD verhindert werden. Interessanterweise waren jedoch lebende BCG in der Lage, die allergische Th2 Immunreaktion zu unterdrücken. Ebenso war die Wirkung von lebendem BCG unabhängig vom IL-10, TLR-2, TLR-4 oder MyD88 vermittelten Signalweg. Wurden Mäuse, die mit den verschiedenen Adjuvantien zusammen mit OVA/alum geimpft wurden, einer zweiten Runde OVA/alum Sensibilisierung unterzogen, so konnten nur lebende und hk-BCG die Entwicklung der Entzündung in der Lunge effektiv unterdrücken. Diese Wirkung konnte durch den adoptiven Transfer von $CD4^+$ T Zellen auf naive Mäuse übertragen werden. Zusammenfassend zeigen diese Daten, daß lebende BCG am effektivsten, gefolgt von hk-BCG, CpG und schließlich PPD allergische Th2 Immunreaktionen unterdrücken konnten.

Als nächstes wurde untersucht, ob eine Impfung mit dendritischen Zellen (DC) die Entwicklung von Th2 Zellen durch die Induktion von allergenspezifischen Th1 Zellen

verhindern kann. Die Applikation von OVA-gepulsten aus dem Knochenmark stammenden-dendritischen Zellen (BM-DC), die mit CpG *in vitro* stimuliert wurden, konnten die Lungeneosinophilie und Entzündung in den Atemwegen in OVA-immunisierten Mäusen nicht reduzieren. OVA-spezifische IgG1 und IgE Antikörpermengen im Serum waren ebenfalls nicht vermindert. Versuche mit OVA-gepulsten Langerhans-zellen (LC) führten zu ähnlichen Ergebnissen wie mit BM-DC. Jedoch waren in Mäusen, die mit CpG/OVA gepulsten BM-DC behandelt wurden, deutlich erhöhte Werte an OVA-spezifischen IgG2a Antikörper im Serum nachzuweisen, was auf die Induktion einer allergenspezifischen Th1 Immunreaktion *in vivo* schließen läßt. Insgesamt zeigen die Ergebnisse aber, dass weder die Impfung mit OVA-gepulsten und CpG-stimulierten BM-DC noch mit OVA-gepulsten LC eine Verringerung der allergischen Th2 Immunreaktion in einem Mausmodell mit schwerem atopischem Asthma bewirkt.

Im dritten Teil der Arbeit wurde NES, ein exkretorisches/sekretorisches Produkt des Helminthen *Nippostrongylus brasiliensis*, als ein neues mögliches Adjuvant zur Unterdrückung allergischer Reaktionen untersucht. Die Applikation von NES zusammen mit OVA/alum inhibierte deutlich die Entwicklung der Lungeneosinophilie, Becherzellmetaplasie und Schleimproduktion in der Lunge sowie die Entwicklung der AHR. Das verwendete NES enthielt geringe Mengen an LPS, die diese Wirkung erklären könnte. Allerdings war die Unterdrückung der Th2 Immunreaktion durch NES unabhängig von TLR-4 und konnte immer noch nachgewiesen werden, wenn LPS-depletiertes NES verwendet wurde. Schließlich konnte NES die OVA-induzierte Th2 Immunreaktion unabhängig von IL-10 und IFN-γ reduzieren. Außerdem konnte der Verdau von NES mit Proteinase K oder eine Hitzebehandlung (kochen) den Th2-unterdrückenden Effekt nicht aufheben. Interessanterweise inhibierte NES *in vivo* eine OVA-spezifische Th2 Immunreaktion in Anwesenheit einer starken NES-spezifischen Th2 Reaktion. Zusammenfassend führen diese Ergebnisse zu dem Schluß, daß der Helminth *N. brasiliensis* Substanzen produziert, die die

Entwicklung von allergischen Th2 Immunreaktionen beeinflussen. Diese Produkte und ihre Wirkmechanismen genauer zu charakterisieren, könnte zu sehr effektiven Adjuvantien führen, welche allergische Reaktionen unterdrücken könnten. Die Ergebnisse dieser Arbeit könnten zukünftig dazu beitragen, effiziente Impfungen zu entwickeln, die Menschen vor der Entwicklung von allergischen Immunreaktionen schützen.

9. ABBREVIATIONS

Ab	Antibody
AHR	Airway hyperreactivity
ANOVA	Analysis of variance
APC	Antigen presenting cells
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette-Guerin
BM-DC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
BSS	Balanced salt solution
CCR	CC chemokine receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
CFU	Colony forming units
CpG-ODN	CpG oligodeoxynucleotides
Ctrl	Control
DC	Dendritic cells
DC-SIGN	DC-specific ICAM-3-grabbing nonintegrin
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FcεRI	Fc epsilon receptor I

FcγRIII	Fc gamma receptor III
FITC	Fluorescein isothyocyanate
GM-CSF	Granulocyte/macrophage colony-stimulating factor
H&E	Haematoxylin and Eosin staining
hk-BCG	Heat-killed BCG
hsp	Heat shock protein
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
IL-10R	IL-10 receptor
i.n.	Intranasal
i.p.	Intraperitoneal
ISAAC	International study of asthma and allergies in childhood
i.v.	Intravenous
LC	Langerhans cells
LPS	Lipopolysaccharide
lyso-PS	Lysophosphatidylserine
mAb	Monoclonal antibody
ManLAM	Mannose-capped lipoarabinomannan
MCPs	Macrophage chemoattractant proteins
MDC	Macrophage-derivated chemokine
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor-88
MLN	Mediastinal lymph nodes
MPL	Monophsphoryl lipid A
mRNA	Messenger RNA

NbCys	NES-derived cysteine protease inhibitor
n.d.	Not detectable
NES	*N. brasiliensis* excretory-secretory products
NeMacs	Nematode-elicited macrophages
NF-κB	Nuclear factor kappa B
NK	Natural killer
OD	Optical density
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid-Shiff reagent
PBS	Phosphate-buffered saline
pDC	Plasmocytoid dendritic cell
PE	Phycoerythrin
PK	Proteinase K
PK NES	PK-digested NES
PPAR-γ	Peroxisome proliferator-activated receptor γ
PPD	Purified protein derivative from *M. tuberculosis*
RNA	Ribonucleic acid
RPA	RNAse protection assay
RT	Room temperature
s.c.	Subcutaneous
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEA	*S. mansoni* egg antigen
SIT	Specific immunotherapy
SP-A	Surfactant-associated protein A

STAT-6	Signal transducer and activator of transcription 6
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tr cell	T regulatory cell
UK	United Kingdom
US	United States of America
VCAM-1	Vascular cell-adhesion molecule-1

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PUBLICATIONS AND ORAL PRESENTATIONS DERIVED FROM THIS WORK

1. Original articles

Erb KJ, **Trujillo C**, Fugate M, Moll H. 2002. Infection with the helminth *Nippostrongylus brasiliensis* does not interfere with the efficient elimination of *Mycobacterium bovis* BCG from the lungs of mice. *Clin Diagn Lab Immunol* 9: 727-730.

Trujillo C, Erb KJ. 2003. Inhibition of allergic disorders by infection with bacteria or the exposure to bacterial products. *Int J Med Microbiol* 293: 123-131. (Review)

Wohlleben G, **Trujillo C**, Müller J, Ritze Y, Grunewald S, Tatsch U, Erb KJ. 2004. Helminth infection modulates the development of allergen-induced airway inflammation. *Int Immunol* 16:1-12.

Trujillo-Vargas CM, Ramírez-Pineda JR, Palmetshofer A, Moll H, Berberich C, Erb KJ. 2005. Mice vaccinated with allergen-pulsed myeloid dendritic cells or Langerhans cells are not protected from developing allergen-specific Th2 responses. *Int Arch Allergy Immunol* (In press).

Trujillo-Vargas CM, Mayer KD, Bickert T, Palmetshofer A, Grunewald S, Ramírez-Pineda JR, Polte T, Hansen G, Wohlleben G, Erb KJ. 2005. Vaccination with Th1 directing adjuvants have different suppressive effects on the development of allergen-induced Th2 responses. *Clin Exp Allergy* (In revision).

Trujillo-Vargas CM, Polte T, Erb KJ. 2004. *Nippostrongylus brasiliensis* excretory-secretory components (NES) inhibit the induction of allergic asthma in the murine model (Manuscript in preparation).

2. Posters and oral presentations at Congresses and Symposia

Trujillo CM, Tatsch U, Erb KJ. The potential use of mycobacterial products as components of vaccines against atopic disorders.

- 33rd Annual Meeting of the German Society of Immunology. Marburg (Germany), Sept. 22-25th, 2002. (Poster)
- Symposium “Th1/Th2 Forschung in Deutschland“. Marburg (Germany), June 26-27th, 2003. (Oral presentation)
- 3rd Joint Retreat GK 520 “Immunomodulation” (Würzburg) and GK 592 “Lymphocyte Activation” (Erlangen), Markt Taschendorf (Germany), Aug. 6-8th, 2003. (Oral presentation)

Trujillo-Vargas CM, Tatsch U, Erb KJ. Vaccination with Th1 directing adjuvants have different suppressive effects on the development of allergen-induced Th2 responses.

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Trujillo-Vargas CM, Polte T, Hansen G, Erb KJ. Nippostrongylus brasiliensis excretory-secretory components (NES) inhibit the induction of allergic asthma in the murine model.

- Join Annual Meeting of the German and Dutch Societies for Immunology (JAMI). Maastricht (Holand), Oct. 20-23rd, 2004. (Oral presentation)
- 17. Mainzer Allergie-Workshop. Mainz (Germany), March 11-12th, 2005. (Oral presentation)

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International Symposium “Frontiers in allergy and autoimmunity”, Mainz (Germany), May 21-22nd, 2004.

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Publications:

Trujillo CM, Ramírez R, Vélez ID, Berberich C. 1999. The humoral immune response to the kinetoplastid membrane protein-11 in patients with American Cutaneous Leishmaniasis and Chagas Disease: prevalence of IgG subclasses and mapping of epitopes. *Immunol Lett* 70: 203-09.

Trujillo CM, Robledo SM, Franco JL, Vélez ID, Patino PJ. 2002. Endemically exposed asymptomatic individuals show no increase in the specific *Leishmania (Viannia) panamensis*-Th1 immune response in comparison to patients with localized cutaneous leishmaniasis. *Parasite Immunol* 24: 455-462.

Erb KJ, **Trujillo C**, Fugate M, Moll H. 2002. Infection with the helminth *Nippostrongylus brasiliensis* does not interfere with the efficient elimination of *Mycobacterium bovis* BCG from the lungs of mice. *Clin Diagn Lab Immunol* 9: 727-730.

Trujillo C, Erb KJ. 2003. Inhibition of allergic disorders by infection with bacteria or the exposure to bacterial products. *Int J Med Microbiol* 293: 123-131. (Review)

Wohlleben G, **Trujillo C**, Müller J, Ritze Y, Grunewald S, Tatsch U, Erb KJ. 2004. Helminth infection modulates the development of allergen-induced airway inflammation. *Int Immunol* 16:1-12.

Trujillo-Vargas CM, Ramírez-Pineda JR, Palmetshofer A, Grunewald S, Moll H, Berberich C, Erb KJ. 2005. Mice vaccinated with allergen-pulsed myeloid dendritic cells or Langerhans cells are not protected from developing allergen-specific Th2 responses. *Int Arch Allergy Immunol* (In press).

Trujillo-Vargas CM, Mayer KD, Bickert T, Palmetshofer A, Grunewald S, Ramírez-Pineda JR, Polte T, Hansen G, Wohlleben G, Erb KJ. 2005. Vaccination with Th1 directing adjuvants have different suppressive effects on the development of allergen-induced Th2 responses. *Clin Exp Allergy* (In revision).

Trujillo-Vargas CM, Polte T, Erb KJ. 2005. *Nippostrongylus brasiliensis* excretory-secretory components (NES) inhibit the induction of allergic asthma in the murine model (Manuscript in preparation).

Eidesstattliche Erklärungen

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation in allen Teilen selbständig angefertigt und keine anderen als die genannten Quellen und Hilfsmittel verwendet habe.

Weiterhin versichere ich, dass ich bisher keinen Promotionsversuch unternommen oder die vorliegende Dissertation weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt habe.

Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften an der Bayerischen Julius-Maximilians-Universität Würzburg.

Würzburg, den 06.04.2005

Claudia Milena Trujillo-Vargas