IMMUNOTHERAPY OF ALLERGIC DISORDERS IN A MOUSE MODEL OF ALLERGIC AIRWAY INFLAMMATION

(Immuntherapie allergischer Erkrankungen in einem Mausmodell für allergische Atemwegsentzündungen)

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der Bayerischen Julius-Maximilians-Universität Würzburg

Vorgelegt von

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Würzburg, 2006

Eingereicht am:

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Tag der mündlichen Prüfung:

Doktorurkunde ausgehändigt am:

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CONTENT

1. INTRODUCTION

1.1. History and prevalence of Allergy

The term allergy was originally defined by Clemens Von Pirquet (1874-1929) as ''an altered capacity of the body to react to a foreign substance,'' which was an extremely broad definition that included all immunological reactions. Allergy is now defined in a much more restricted manner as ''disease following a response by the immune system to an otherwise innocuous antigen'' and refers to IgE-mediated immediate hypersensitivity reactions of the immune system to specific substances called allergens (such as pollen, insect venom, drugs or food). As many as 40% of people in Western populations show an exaggerated tendency to mount IgE responses to a wide variety of common environmental allergens. This state is called atopy and seems to be influenced by several genetic loci (Janeway et al., 2001).

The existence of allergic reaction can be documented by the measurement of IgE antibodies to specific allergens in the blood (Radioallergosorbent test - RAST) and by the use of skin prick test (evaluating immediate hypersensitivity reactions after epicutaneous application of the allergen).

Allergic diseases such as asthma, allergic rhinitis, atopic dermatitis and food allergy afflict up to 20% of the human population in most countries and are believed to be increasing in prevalence (Kim H. et al., 2001). More often allergic reactions occur in the gastrointestinal and respiratory tract due to the direct interaction with environmental allergens. Inhalation is the most common route of allergen entry causing allergic rhinitis (hay fever), allergic conjunctivitis and allergic asthma. Allergic rhinitis and conjunctivitis are commonly caused by environmental allergens that are only present during certain seasons of the year (certain grass and tree pollen).

A more serious syndrome is allergic asthma, which is triggered by allergen-induced activation of submucosal mast cells in the lower airways (Janeway et al., 2001). Allergic asthma is a chronic inflammatory disease of the respiratory tract with rising incidence and prevalence in industrial countries. According to the World Health Organization, asthma affects 150 million people worldwide and is the most prevalent chronic disease of childhood (Epstein M., 2004). Asthma morbidity and mortality have increased over the last two decades, particularly in western countries (Beasley et al., 2000). Among children, higher prevalence rates have been found in industrialized Western countries than in developing countries in Asia and Africa (Beasley et al., 2000). In the Middle East, asthma prevalence is reported to be lower than in developed countries (Behbehani et al., 2000). The International Study of Asthma and Allergies in Childhood (ISAAC) was the first study carried out worldwide using standardized questionnaires in order to create a reliable global map of childhood allergy. They studied 257.800 children aged 6 to 7 years in 38 countries, and 463.801 children aged 13 to 14 years 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 - 14.9% in the 6-7 year-olds and from 1.4 – 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 Committee, 1998). 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 (Rodrigo et al.,2004). It is estimated that there are 18 asthma-related deaths per million people and 180,000 deaths per year (WHO, 2000).

High prevalence and increasing evidence of allergic asthma in the last decades underlines the importance to understand the pathogenesis of disease in order to develop novel therapeutic interventions (Hahn et al., 2003).

1.2. Hypothesis explaining the increasing prevalence of asthma

Although the immunological processes leading to the development of allergic disease 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 and 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. (Skadhauge et al., 1999). Individuals, with two atopic parents are at great risk of developing an allergic disease than those with only one atopic parent (approximately 47% versus 24%), but the specific allergic disease in those individuals may be different from those of the parents (Paul, 1997).

The association of genes and their polymorphisms with features of asthma has been an important advance over the past decade (Bochner et al., 2005). Several genomic regions are reported to be associated with the development of asthma and allergy (Munthe-Kaas et al., 2004). Recent studies evaluated the genetic linkage between asthma phenotypes to chromosome 20p13, which was described as an asthma susceptibility gene by Van Eerdewegh et al., 2002, (Raby et al., 2004).

Munthe-Kaas et al (2004) reported the association of the development of allergy and asthma with chromosome 2q33, which contains the candidate gene cytotoxic T-lymphocyte antigen (CTLA-4). Based on there findings, the authors summarized that a role for CTLA-4

polymorphisms exists in determining the Th1/Th2 balance and identifying CTLA4 signaling as a potential target in atopic disease.

In addition the Th2 cluster on human chromosome 5q23-35, containing loci for the Th2 cytokines IL-4, IL-5, IL-9, IL-13 and other potentially relevant genes, has been shown by various studies to have a significant linkage with total IgE levels (Marsh DG et al., 1994).

The investigation of Toll-like receptor (TLR) genotypes revealed the possibility of involvement of genetic variations in TLR2 as a major determinant in the susceptibility to asthma and allergies in children of farming families (Eder et al., 2004). Furthermore, studies on TLR4 and CD14 gene polymorphism evaluated, that decreased IL-12 and IL-10 production was associated with a TLR4 polimorphism (Asp299Gly) (Fageras et al., 2004). Because the generation of these particular cytokines might be important in downregulating the Th2 response, deficiency in this response might explain how innate immune responses could be important determinants of allergy and influence the outcome of intervention studies that use microbial stimuli as immune modulators (Bochner et al., 2005).

Whereas the role of heritability in the development of atopic disease is documented, it is not able to explain the ''epidemy of atopy'' that has been observed over the past three decades. The reason for this phenomenon is still not known, however, there are several possible explanations for the increase in allergic asthma.

First, it is possible that the genetic background of the human population living in developed versus developing countries is different, resulting in 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). 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 consequently, 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).

Secondly, epidemiological studies indicate that the increased prevalence of allergic diseases is associated with a reduced microbial stimulation of the immune system (Boini, 2005). The hygiene hypothesis, proposed by Strachan 1989, states that a reduced exposure to allergens in early life is solely implicated in the growing propensity for allergy sensitization. Important elements of the hypothesis include helminth infection, exposure to endotoxins, exposure to pets and growing up on a farm (Platts-Mills et al., 2005). 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) and modulates so called Th1/Th2 balance (Matricardi et al., 2002). 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 (Parronchi et al., 1992) (Lack et al., 1996) (Li et al., 1996). Recent studies demonstrated 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 disease and 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. This stimulates the production of proinflamatory 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 as the influence of LPS exposure on the development and maintenance of allergic diseases. Several studies reported the influence of Bacille Calmette-Guerin (BCG) vaccination on the reduction of atopic disease (Shirakawa et al., 1997). BCG inoculation in mice, delivered 14 days before allergen sensitization, reduced the formation of specific IgE in response to allergen, with an increase in production of IFN-y (Herz et al., 1998). Similar results have been obtained in mice with the a single injection of heat-killed *Mycobacterium vaccae,* another potent inducer of Th1 responses (Wang et al., 1998), and with *Listeria*.

Finally, over the last half of the 20th century, there have been major changes in diet and physical activity. There are three distinct, but strongly interrelated, aspects of lifestyle that could be relevant to the prevalence and severity of asthma: diet, physical activity and obesity (Platts-Mills et al., 2005). The possibility that obesity is related to asthma was first suggested in 1994, and there have been multiple reports of an association between an elevated body mass index (BMI) and asthma since then (Luder et al., 2004). The most obvious one is that it is much easier to document BMI than diet or physical activity. However, although some of the obesity data are convincing, they are not consistent, and the association between obesity and asthma is certainly not comparable with obesity and diseases such as childhood type 2 diabetes (Platts-Mills et al., 2005). In a typical study, the prevalence of wheezing was 13%

among those in the quintile with the highest BMI and 7% among those in the lowest BMI quintile (Berkey et al., 2000).

Further, several studies concluded that full expansion of the lungs had a more potent effect on bronchial smooth muscle than isoprenaline (beta-adrenergic agonist which has a powerful stimulant action on the heart, increasing cardiac output and heart rate). Additionally, prolonged shallow breathing (20min) can lead to increased non-specific bronchial reactivity (Fredberg et al., 1999) (Skloot et al., 1995).

1.3. Molecular and Immunological mechanisms of asthma

Asthma is a chronic inflammatory condition of the airways that is characterized by a prominent eosinophilic inflammatory infiltrate in the bronchial mucosa. (Flood-Page et al., 2003). 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). Allergens entering the body by way of the respiratory or digestive tracts or through the skin (Kidd, 2003). The immune system is designed to either respond or tolerate foreign antigens. In atopic individuals non-pathogenic foreign proteins elicit an allergic class of response (Epstein M., 2004).

In pre-disposed individuals, initial exposure to allergen leads to the activation of allergen-specific T helper 2 (Th2) cells and IgE synthesis, which is known as allergic sensitization (Figure 1). Subsequent exposure to allergen cause inflammatory-cell recruitment and activation and mediator release, which are responsible for early (acute) allergic responses (EARs) and late allergic responses (LARs). In the EAR, within minutes of contact with allergen, IgE-sensitized mast cells degranulate and release both pre-formed and newly synthesized mediators in sensitized individuals. These include histamine, leukotrienes and

cytokines, which promote vascular permeability, smooth-muscle contraction and mucus production. Chemokines released by mast cells and other cell types direct recruitment of inflammatory cells that contribute to the LAR, which is characterized by an influx of eosinophils and Th2 cells. Eosinophils release an array of pro-inflammatory mediators, including leukotrienes and toxic proteins (eosinophil cationic protein, eosinophil peroxidase, major basic protein and eosinophil-derived neurotoxin), and they might be an important source of IL-3, IL-5, IL-13 and granulocyte/macrophage colony-stimulating factor (Hawrylowicz and O'Garra., 2005).

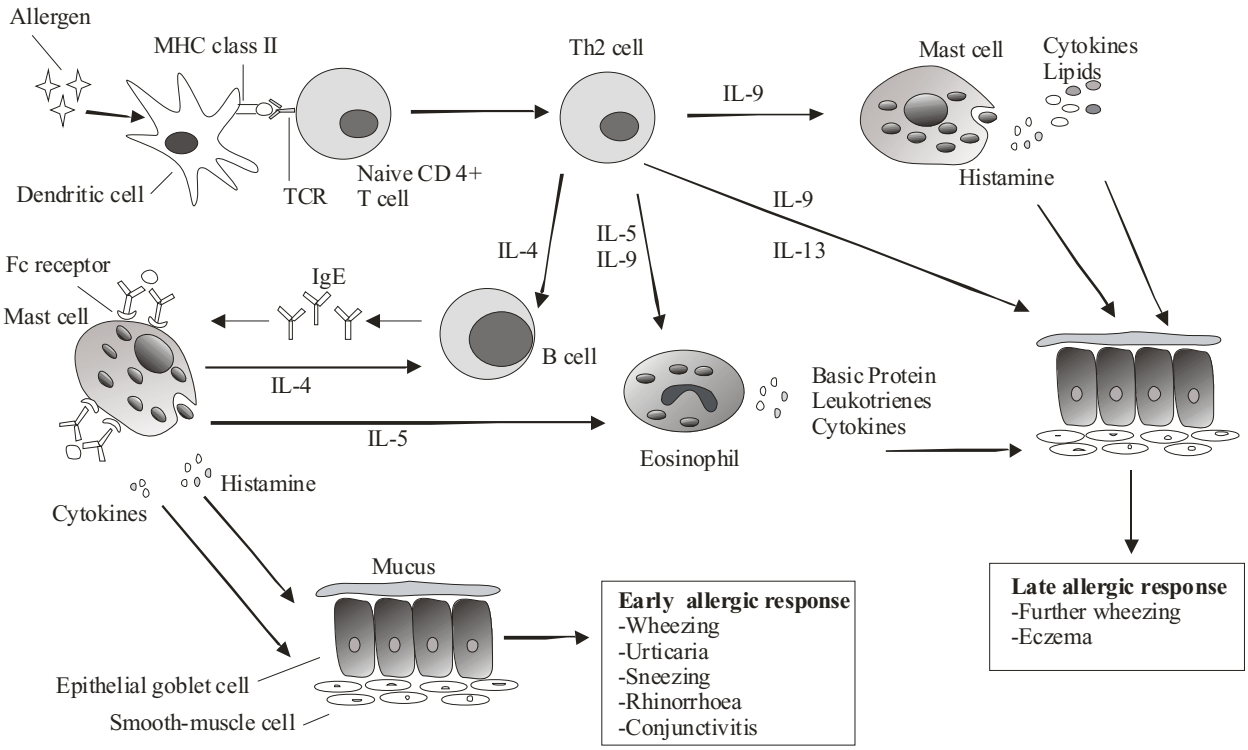


Figure 1. *Mechanism of atopic allergy.*

The initial immunological events in the response to inhaled antigen include antigen uptake by professional APCs, processing into immunogenic peptides and presentation, in the context of major histocompatibility complex (MHC) molecules, to naïve T cells in the lymph nodes that drain the lung. In this regard, pulmonary dendritic cells (DCs) play a critical role in sampling and presenting antigen in the lung, which leads to activation and expansion of CD4+ T cells and preferential production of T helper subset 2 (Th2)-biased adaptive immune responses (Akbari et al., 2001). 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). In this place, 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 all allergens to naïve CD4+ T cells is initiated. Both Th2-biased immune responses as well as immune responses that protect against allergic disease and asthma depend on activation of antigen-specific T cells by APCs (Akbari et al., 2003).

For instance, subset of DCs (DC1) that produce IL-12 and express high levels of costimulatory molecules, such as B7.1 and B7.2, induce Th1 response, associated with the production of IFN-γ and IL-2 (Rissoan et al., 1999). In contrast, DC2 subsets associated with the production of IL-4, IL-5 and IL-13 and expressing low levels of costimulatory molecules (Stumbles et al., 1998) (Eisenbarth et al., 2002). Predisposition towards the development of Th2 responses may be genetically determined, and occur more frequently in atopic individuals. In other situations, DCs induce T cell unresponsiveness, anergy or apoptosis (Inaba et al., 1991). Whereas immature DCs may be important in inducing deletional tolerance or anergy, for example, in central tolerance, mature DCs expressing high levels of costimulatory molecules (ICOSL, OX-40L, B7-1, B7-2) may be required for induction of regulatory cells (Akbari et al., 2001) and for induction of peripheral tolerance mediated by

suppression, particularly in response to exogenous environmental allergens (Akbari et al., 2003). The consequence of antigen recognition is activation, differentiation, and clonal expansion of CD4+ T helper 2 (Th2 cells), alteration of the quantity and type of cell surface molecules, production and secretion of IL-4, IL-5, IL-13 cytokines, and generation of cells that can provide help to B lymphocytes (Epstein M., 2004).

1.4. Th2 based pathway leads into the pathogenesis of allergy

Atopic disease is dependent on the production of the cytokines IL-4, IL-5 and IL-13 by allergen-specific Th2 cells that are generated from naive CD4+ precursors (Lewis., 2002) after recognition the allergen-derived peptides on the surface of APC. The recruitment of Th2 cells is also induced after allergen challenge with allergic asthma (Kroegel et al., 1996). 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 naïve mice can induce airway eosinophilia, mucus hypersecretion and AHR after allergen challenge (Cohn et al., 1997).

IL-4 is a essential for Th2-cell development, which is not produced by DC. Potential candidates for the early production of IL-4 include a population of NK1.1+CD4+T cells, which can rapidly produce large amount of IL-4 upon suitable activation in vivo. Alternatively, naïve T cells itself produce IL-4 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 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). Upon activation, this protein dimerizes and translocates 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. GATA-3 directly transactivates the IL-5 promoter, inducing IL-5 production. In addition, GATA-3 seems to be a chromatin remodelling 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). IL-4 and IL-13, two related cytokines with overlapping functions, are considered to be key players in IgE class switching. Both cytokines share the IL-4Rα subunit in their receptor complex. IL-4Rα$^{-/-}$ mice are strongly impaired in type 2 T-helper cell (Th2)-dependent immune reactions, and cells driven from these mice completely fail to respond to IL-4 or IL-13, which suggests that neither cytokines has functional receptor lacking this chain (Barner et al., 1998)(Mohrs et al., 1999). Class switching to IgE, and to IgG1, in mice is induced when B cells simultaneously receive two stimuli: one is the activation of the shared IL-4/IL-13 receptor by one of its ligands, and the other is the ligation of CD40 by CD40L, which is expressed on activated T cells (Worm et al., 1997)(Bacharier et al., 1998). IgE 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). Binding of IgE to FcεRI renders mast cells and basophils ``sensitized`` to a new challenge with the allergen. Upon secondary exposure with multivalent allergen, the crosslinking of FcεRI–bound IgE initiates the activation of mast cells and basophils by promoting the aggregation of FcεRI. Initially, IL-4 was believed to be exclusively responsible for the class switch to IgE in the mouse. The discovery that IL-13 can also induce IgE class switching in human B cells

(Punnonen et al., 1993)(Defrance et al., 1994) suggested a possible role for IL-13 in mice as well. IL-13-induced IgE production was indeed demonstrated in transgenic mice which expressed IL-13 in lymphoid tissues, but lacked a functional IL-4 gene (Emson et al., 1998). Beside of contribution to isotype switching in B cells, investigations on overexpression of IL-13 in the lung and in acute models of asthma in mice have defined important roles for IL-13 in the induction of mucus hypersecretion, goblet cell hyperplasia, subepithelial fibrosis, eotaxin production, eosinophil accumulation and the promotion of enhanced airways hyperreactivity(AHR) to cholinergic stimuli (Foster et al., 2003).

The role of IL-4 in allergic inflammation is not limited, 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 (Mochizuki et al., 1998). In addition, IL-4 acts directly on lung fibroblasts inducing a fibrogenic responses, which further amplify 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 and IL-9. IL-5 is a key cytokine in eosinophil differentiation and maturation in the bone marrow as well as in recruitment and activation at sites of allergic inflammation. (Flood-Page et al, 2003). IL-5 stimulates the expansion and differentiation of eosinophil precursors, upregulates expression of its own specific receptor α chain during human eosinophil development, (Tuvernier J et al, 2000) primes eosinophils for enhanced chemotaxis and hyperadherence and delays apoptosis. (Sehmi R et al., 1992). In

animal studies, monoclonal antibodies (mAbs) directed against IL-5 have been shown to suppress airway eosinophilia and airway hyperresponsiveness (AHR)(Matsumoto et al., 2003). Genetically IL-5-deficient mice have been shown to fail to develop airway eosinophilia and AHR after allergen challenge (Foster et al., 1996). Moreover, adoptive transfer of IL-5-producing CD4+ Th2 type cells resulted in airway eosinophilia and AHR after allergen challenge in IL-5-deficient mice (Hogan et al., 1998).

IL-9 is another Th2-derived cytokine, which is thought to play an important role in th e development of asthma. Evidence from both murine and human studies shows the IL-9 gene to be located within an area of the chromosome associated with susceptibility to AHR (Postma et al., 1995)(Nicolaides et al., 1997). The biological effects of IL-9 are pleiotropic, as it acts as a growth factor for mouse T cells, a maturation factor for B cells (Vink et al., 1999) and proliferation factor for mast cells and hematopoietic progenitors (McMillan et al., 2002). In allergy, the expression of IL-9 and its receptor is increased and this increase correlates to changes in lung function (Shimbara et al, 2000). Eosinophils also synthesize and secrete IL-9 and evidence suggests that IL-9 may potentiate eosinophil function in vivo via interactions with IL-5. IL-9 has been found to increase eosinophil survival, as well as IL-5-mediated differentiation and maturation (Louahed et al., 2001). In another in vitro study, IL-9 was found to stimulate mucin production in respiratory epithelial cells (Longphre et al., 1999).

The method of targeting T cells to treat allergic asthma is an interesting strategy that has not yet been explored extensively. In contrast to current therapies, some T-cell directed therapies harbour the potential to induce long-lasting suppression or even complete remission of disease.

1.5. Therapeutic strategies for allergic disease

Several potential points of therapeutic intervention are proposed for the treatment of allergic diseases (Figure 2). One possibility is to block activation of allergen-specific Th2 cells, either directly or indirectly through effects on antigen-presenting cells: for example by treatment with anti-inflammatory drugs, such as glucocorticoids, or by allergen immunotherapy. Another option is to block effector molecules that cause the clinical symptoms of allergic disease: for example, by treatment with antihistamines, leukotriene antagonists, neutralizing antibodies specific for Th2 cytokines or antibodies specific for IgE.

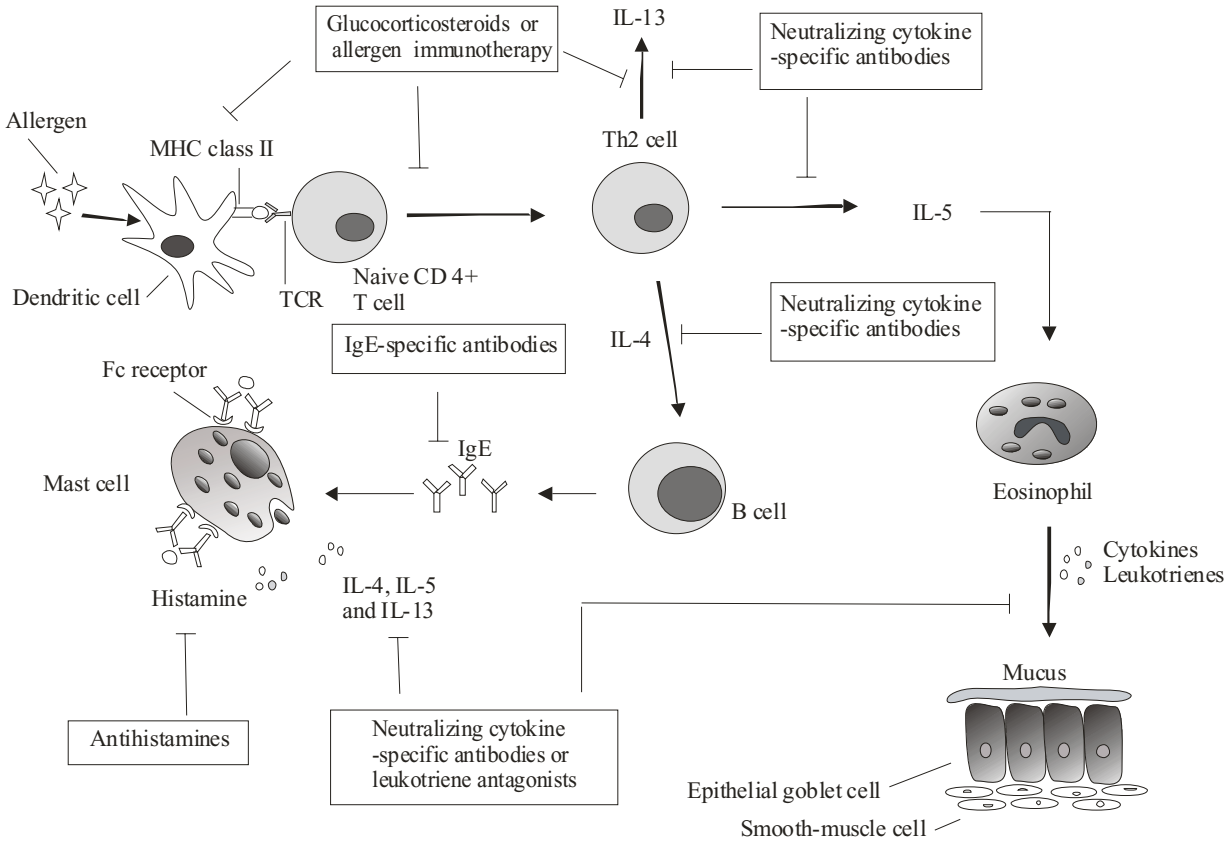


Figure 2. *The allergic pathway and potential points of intervention.*

Current treatments for allergy and asthma are mainly based on pharmacological interventions, such as treatment with antihistamins, glucocorticoids or β-agonists (Holgate et al., 2003)(Barnes , 1997). Although these treatments are highly effective for controlling disease in most individuals, many patients must take these drugs for life.

Corticosteroids are the one of the most effective treatments currently available for atopic diseases and high doses of oral corticosteroids would control almost every atopic patient. However, systemic side effects limit the dose that can be given over long periods, and this led to the development of topical steroids. There is little doubt that inhaled corticosteroids have revolutionized the treatment of asthma and are now first-line treatment for chronic asthma in patients of all ages and severity of disease (Barnes et al., 1998). Herewith, beside of efficiency they do not cure disease and allergic inflammation recurs when treatment is stoped. Advances in understanding how corticosteroids suppress inflammation at a molecular level may lead to the development of safer steroids, or drugs that mimic their key anti-inflammatory actions. Corticosteroids bind to a cytosolic glucocorticoid receptor which translocates to the nucleus and binds as a homodimer to DNA to activate genes. The principal action of corticosteroids is to suppress multiple inflammatory genes, including cytokines, inflammatory enzymes, adhesion molecules and inflammatory mediator receptors, and this is why corticosteroids are so effective in complex inflammatory conditions (Barnes., 1999). However, steroids show more immediate effects and also induce long-lasting changes in the differentiation of T cells. Soon (3-4 h) after systemic steroid administration, glucocorticoids induce a redistribution of cells, and there effectiveness has been shown to be attributed with reduced activation of peripheral T cells, as well as IL-4 and IL-5 expression (Gemou-Engesaeth et al., 2002)(Bentley et al., 1996). This redistribution also affects T regs, which are found more frequently in the peripheral blood following steroid treatment. Significantly increased FOXP3

mRNA expression was found in unstimulated peripheral blood CD4+ T cells of both patients with severe asthma treated with systemic glucocorticoids and patients with moderate asthma treated with inhaled glucocorticoids but not in patients with untreated moderate asthma. The higher T reg activity in glucocorticoid-treated patients with asthma is demonstrated by increased IL-10 mRNA expression, which tightly correlated with the FOXP3 expression (Karagiannidies et al., 2004).

Antihistamins are effective in rhinitis and reduce itch in atopic dermatitis, but have no clear benefit in asthma (Van Ganse et al., 1997). New antihistamins have been claimed to have additional anti-asthma effects that are not mediated through H_1-receptor blockade. These effects include an inhibitory effect on eosinophil chemotaxis and adherence to endothelial cells, and inhibition of eosinophil recruitment into asthmatic airways after allergen challenge (Barnes., 1999).

Cysteinyl leukotrienes, generated from the rate-limiting enzyme 5´- lipoxygenase (5-LO), are potent bronchoconstrictors and inducers of plasma exudation, and there is some evidence that they may promote eosinophilic inflammation. 5-LO inhibitors and Cysteinyl-leukotriene receptor (Cys-LT_1) antagonists have been developed for the treatment of asthma, and possibly other atopic diseases. In challenge studies they reduce allergen- and exercise-induced asthma, as well as several other challenges. In clinical trials they improve asthma symptoms, lung function and reduce the need for rescue bronchodilator treatment (Drazen et al., 1999).

A monoclonal anti-IgE antibodies – Omalizumab, was first humanized therapeutic antibody approved by FDA (U.S. Food and Drug Administration, 2003) for treatment of asthma and the first approved therapy designed to target immunoglobulin E (IgE) (Curtiss., 2005). Omalizumab targeting the high-affinity receptor binding site on human IgE. Bound IgE is not available for basophil binding, degranulation is attenuated, and allergic symptoms are

reduced. In asthma trials, omalizumab reduced inhaled corticosteroid and rescue medication requirements and improved asthma control and asthma quality of life in moderate to severe allergic asthmatics with disease poorly controlled by inhaled corticosteroids (Belliveau., 2005).

1.6. Neutralizing strategies of Th2 type cytokines

Different therapeutic strategies that aim to neutralize Th2-type cytokines or to restore the dysregulated Th2-dominated allergic reactions are currently in (pre)clinical development (Heijink and Oosterhout., 2005). There are several possible approaches to inhibit specific cytokines. These include the use of drugs that inhibit cytokine synthesis, humanized blocking antibodies to cytokines or their receptors, soluble receptors that `mop up´ secreted cytokines to receptor antagonists and drugs that block the signal-transduction pathways activated by cytokines. On the other hand, there are cytokines that suppress the allergic inflammatory process and these may have therapeutic potential.

As asthmatics have elevated IL-5 protein levels in serum and marked eosinophil infiltration in bronchial biopsies, removing IL-5 is a proposed therapy for asthma. In support of a role for IL-5 in asthma, various experimental studies have shown a reduction in pulmonary inflammation, including eosinophilia, in IL-5-deficient mice or animals treated with neutralising anti-IL-5 monoclonal antibodies (Foster et al., 1996)(Hamelmann et al., 1997).

Despite cumulative evidence for a therapeutic role for IL-5 depletion in asthmatics, clinical administration of a humanized monoclonal antibody against IL-5 in mild asthmatics show no effect on airway hyperresponsiveness (Leckie et al., 2000). More recent study confirmed that anti-IL-5 treatment did not significantly alter clinical parameters in mild asthmatics (Flood-Page et al., 2003).

Inhibition of IL-4 may be helpful in type I allergic reactions, where IL-4 is not only critically important for Th2 cell differentiation at the beginning of an immune response, but is also responsible for downstream events leading to differentiation and activation of effector cells (Mueller et al., 2002). Potential selective IL-4 inhibitors are the soluble extracellular domain of the IL-4 receptor alpha chain (sIL-4Rα) or antibodies to IL-4 (Renz et al., 1996)(Henderson et al., 2000). Both have been shown to inhibit allergen-specific IgE, airway eosinophilia, and AHR to methacholine, when given during sensitization in mice. In contrast, the eosinophilic inflammation could be reduced only marginally and AHR was not affected when IL-4 antagonists were applied during allergen challenge (Coyle et al., 1995). However, it was recently reported that inhalation of sIL-4Rα ameliorated asthma scores in patients with moderate asthma during phase I/II clinical studies (Borish et al., 2001).

A selective IL-13 inhibitor on the basis of a soluble hIL-13R fusion protein (sIL-13Ra2-FC) or antibodies against IL-13 have been shown to inhibit the development of AHR, goblet cell metaplasia, and eosinophilic inflammation in a murine model for allergic asthma, when applied during allergen challenge (Wills-Karp et al., 1998)(Blease et al., 2001).

Additionally, various studies have further illustrated redundancy and overlap in function between the four major (IL-4, IL-5, IL-13 and IL-9) Th2 cytokines (Fallon et al., 2002), and it has been suggested that a single cytokine antagonists might have limited efficacy (Hahn et al., 2003).

IL-4 and IL-13 share one common receptor subunit, the IL-4 receptor alpha chain (IL-4Rα chain), therefore the blocking of IL-4Rα would lead to a complete inhibition of both IL-4 and IL-13 signalling (Duschl,. 2000). Indeed, mice lacking a functional gene for this receptor subunit fail to respond to either cytokine and are selectively impaired in Th2-associated

immune responses in models of parasite infections and allergies (Urban et al., 1998)(Mohrs et al., 1999) (Cohn et al., 1999)(Noben-Trauth et al., 1999).

Previously, a highly efficient murine IL-4 antagonist (QY) has been developed (Grunewald et al., 1997), where the amino acids glutamine 116 and tyrosine 119 were mutated to aspartic acid. This murine mutant is analogous to the R121D/Y124D double mutant of human IL-4. QY binds with high affinity to the murine IL-4Rα without inducing signal transduction (Figure 3), has no detectable activity upon proliferation or differentiation of murine cells, and an excess of QY completely inhibits responses toward wild-type IL-4. Like its human analogue, the QY mutant is an antagonist for IL-13 (Grunewald et al., 1997). Recent experiments with monocytic cells from mice lacking a functional γc gene showed, that QY is a complete inhibitor for IL-4 in the absence of γc as well (Andersson et al., 1997).

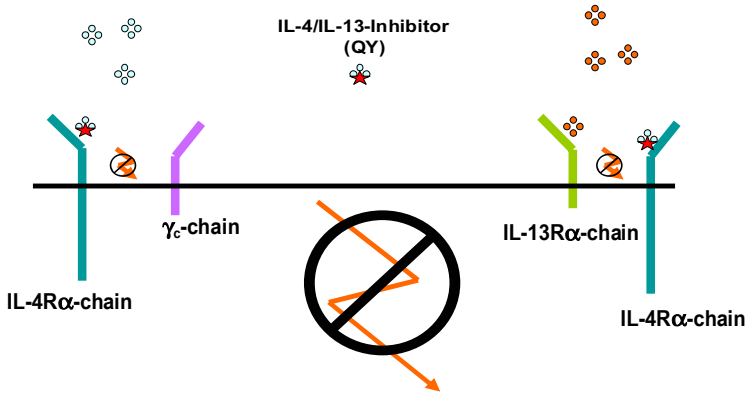


Figure 3. *Inhibition of the IL-4/IL-13 system with the inhibitory molecule QY.*

Recent studies demonstrate, that use of an IL-4/IL-13 receptor antagonist QY shows a complete inhibition of IL-4 and IL-13 mediated reactions in vitro. In addition, specific IgE synthesis and the development of immediate type hypersensitivity could be inhibited

completely during primary sensitization with ovalbumin (OVA) in mice (Grunewald et al., 1998). In congruence with this study other experiments demonstrated the efficient prevention of the development of an allergic airway condition (Tomkinson et al., 2001). However, a recent study in a murine asthma model showed, that beside of the improvement of the allergic condition after application of IL-4/IL-13 inhibitor during allergic sensitization, the inhibition of an IL-4/IL-13 system after the development of allergic airway pathologic condition did not show any significant reduction of all measured allergic parameters (Hahn et al., 2003).

All of the above mentioned treatments focus on the non-allergen-specific alleviation of symptoms, rather than treating the underlying cause. So far, the only allergen-specific treatment that has the potential for a long-term ´curative´ approach is Allergen Specific Immunotherapy (SIT)(Wachholz and Durham., 2004).

1.7. Mechanism of Allergen Specific Immunotherapy

Traditional SIT has been in use for almost a century and is one of the few specific immunomodulatory treatments that are commonly used for an immune-mediated pathology (Norman., 2004). It involves the injection of increasing doses of specific allergen extract into the patient, and it is highly effective in carefully selected patients who have IgE-mediated disease, such as allergic rhinitis, asthma and venom anaphylaxis. The allergen is generally given subcutaneously, but it can also be administered sublingually or intranasally (Wilson et al., 2003). Recent studies suggest, that SIT not only gives symptom relieve in allergic disease but also modifies the natural course of disease by reducing the risk of developing new allergic sensitizations and inhibiting the development of clinical asthma in children treated for allergic rhinitis (Frew., 2003). Whereas the clinical efficacy of specific immunotherapy is well documented, its molecular mechanisms are incompletely understood.

Successful SIT is associated with decreased ex vivo T cell allergen-specific proliferation, a reduced ratio of IL-4, IL-5 or IL-13 production and decreases the recruitment of effector cells at the site of inflammation (Lewis., 2002) (Till et al., 2004). Moreover, it suppress the antigen specific IgE´s synthesis and increases level of serum allergen-specific IgG1, IgG4 and IgA antibodies (Jutel et al., 2003). Observed increase in IgG4 and decrease in IgE often occur months after a therapeutic response of SIT. This suggests that these alterations are a late maker for successful SIT (Akdis et al., 2001) (Till et al., 2004). IgG4, which has little pro-inflammatory activity, has been proposed to compete with IgE for binding to mast cells and basophils – presumably through competition for allergen binding, although the mechanisms for this are still unclear. IgG4 might also reduce IgE-facilitated allergen presentation by preventing the binding of allergen-IgE complexes to APCs (Wachholz and Durham., 2003)(Wachholz et al., 2003).

Early studies indicated that effective allergen immunotherapy was associated with immune deviation from a disease-promoting Th2 response towards a Th1 response (Jutel et al., 1995) (Ebner et al., 1997) (Wachholz et al., 2002). However, not all reports agree on the increase in IFN-y-associated responses after allergen immunotherapy (Soderlund et al., 1997)(Van Bever et al., 1998)(Klimek et al., 1999). Subsequently, evidence from studies on immune responses to insect venoms, indicates that successful allergen immunotherapy is associated with a decrease in the allergen-specific Th2 response and the induction of allergen-induced IL-10-secreting T cells (Akdis et al., 1998)(Till et al., 2004). Evidence for T-cell production of IL-10 in response to allergen, both in bee-keepers who had become tolerant following repeated insect stings and in non-atopic individuals, has also been used to support the contention that IL-10 regulates tolerance to allergen (Akdis et al., 1998). Furthermore, neutralization of IL-10 in allergen-stimulated peripheral-blood cultures restores production of Th2 cytokines. More

recently, several laboratories have reproduced these findings for both systematically introduced allergens (Faith et al., 2003) and aeroallergens (Francis et al., 2003)(Nouri-Aria et al., 2004)(Oldfield et al., 2002) and have shown an increase in either IL-10 alone or both IL-10 and IFN-y. The IL-10-expressing cells that are detected after allergen immunotherapy have been shown to be CD25+ T cells (Francis et al., 2003) and SIT-induced unresponsiveness could be prevented by depletion of CD25+ T cells in vitro (Jutel et al., 2003). However, it is unclear as yet whether these are naturally occurring Treg cells, or activated effector cells that upregulate CD25 and have deviated from a Th2 phenotype towards an IL-10-secreting phenotype, or a third population that is distinct from either of these. This regulatory T-cell population contains at least some allergen-specific cells, because it responds to allergen during in vitro culturing (Hawrylowicz et al., 2005).

Besides of efficiency SIT has several limitations. The first is the considerable risk of adverse side-effects, including severe anaphylaxis, through the interaction of IgE with the injected allergen. Second, the protocol requires administration of allergen for a sustained period of several years to maximize long-lasting effects (Casale et al., 2004).

Taken together, SIT remains the only curative and non-symptomatic treatment of allergy, and understanding the immune mechanism that underlies successful allergen immunotherapy offers the potential to improve current allergen-immunotherapy regimens.

1.8. Regulatory T cells in allergy

There is increasing evidence that the occurrence of allergic disease itself reflects an imbalance between IL-10-secreting regulatory T cells and Th2 cells. Recent study showed an increased frequency of IL-4-secreting, allergen-specific T cells in atopic patients and an increased frequency of IL-10-secreting, allergen-specific T cells in non-atopic individuals (Akdis et al.,

2004). Moreover, both naturally occurring Treg cells and inducible populations of antigen-specific IL-10 secreting regulatory T cells have been shown to inhibit Th2 responses in experimental allergies (Hawrylowicz et al., 2005). Studies in humans provide evidence that induction of IL-10 synthesis is associated with amelioration of disease symptoms (John M et al., 1998)(Hawrylowicz et al., 2002). Additionally, animal studies described the suppression of allergic airway inflammation resulted by transfer of IL-10 to the lungs and after adoptive transfer of IL-10-transfected T cells (Stampfli et al., 1999)(Oh et al., 2002). Several reports showed that IL-10 modulates many cells and effector functions associated with allergic disease and therefore it has the role in the natural regulation of immune homeostasis in the lungs (Table 1)(Royer et al., 2001)(Jeannin et al., 1998)(Nouri-Aria et al., 2004)(Arock et al., 1996).

Interleukin IL-10 inhibits
➢ Activation of mast cells and there cytokine generation
➢ Survival of eosinophils and there cytokine production
➢ Function of antigen-presenting cells (APC), including the maturation of dendritic cells, the expression of MHC class II and co-stimulatory molecules
➢ Activation of Th2 cells

Table 1. *Functions of IL-10 relevant to allergy and asthma.*

It has been realized that in addition to the recognition of MHC/peptide complexes, the activation of resting T-cells requires a second or ``costimulatory`` signal (Schwartz ., 1990). Costimulatory receptor CD28, expressed on the cell surface of resting and activated T cells and its counterreceptors B7-1(CD80) and B7-2(CD86) expressed on antigen presenting cells (Köhler et al., 1975), in particular on DC, and are further upregulated upon APC activation

(Sharpe AH et al., 2002). While CD28 is constitutively expressed, its inhibitory relative CTLA-4, shares common ligands and is induced as an intracellular protein in response to T cell activation and transported to the immunological synapse where it is though to dominantly inhibit signaling (Chambers et al., 2001).

Recently, it has been reported that stimulation of immune system with a superagonistic mAb to CD28 disproportionately expandes Treg cells (Lin and Hünig., 2003). Authors observed a fourfold higher increase of CD4+CD25+ T cells due to the in vivo application of a CD28 supseragonist, than the increase of CD4+CD25- T cell subsets. Herewith, the expanded population of CD4+CD25+ T cells display all features of Treg cells, suggesting that CD28 superagonistic therapy will be useful for the treatment of a variety of immunological conditions (Lin and Hünig., 2003).

2. AIMS

In order to improve allergy therapy various potential anti-allergic strategies were investigated in this thesis work for therapeutic effects in a mouse model for allergic airway inflammation.

- First a mouse model for SIT was established in order to study the immunological mechanisms underlying successful SIT.
- Since adjuvant therapies are likely to improve the treatment efficacy, strategies combining the SIT with other therapeutic approaches were investigated. Beside the therapeutic effects of SIT alone, the IL-4/ IL-13 system was inhibited by use of an antagonistic IL-4 molecule.
- Furthermore anti CD28 monoclonal antibodies were used in order to induce T regulatory cells in vivo which should promote an anti-allergic effect in mouse model of allergic airway inflammation.

3. MATERIALS AND METHODS

3.1. Animals

Female BALB/c mice between 4-6 weeks of age were purchased from Harlan Winkelman (Borchen, Germany) or Charles River (Sulzfeld, Germany) and were maintaned under specific pathogen-free conditions. At the onset of the experiments, animals were between 6-8 weeks of age. All animal experiments were performed according to the guidelines for the care and use of experimental animals prepared and published by the Society for Laboratory Animal Sciences (GV-SOLAS), Biberach a.d. Riss, 1988.

3.2. Anaesthesia

To perform an allergic sensitization the animals were anaesthetised for 20-30 min with intraperitoneal injection of Ketamine 100mg/ml (Pharmacia)/Xylazin 2% (Bayer) mixture in sterile PBS. The concentration of anaesthetics were 80µg Ketamine and 10 µg Xylazin for one gram of animal body weight. In order to make a read out of experiments mice were subjected to a intraperitoneal injection with a lethal dose, which was 10 times concentrated anaesthetic solution.

3.3. Methods of blood collection

The blood was collected at various time points of sensitization and therapy courses. Therefore mice exposed to an infrared light for 5 min. Then a small incision was performed on the tail vein and blood was collected in tubes. At the end of experiments (24 hours after the last challenge), mice subjected to lethal anaesthesia and bled from axilar vein. Blood was coagulated at 4°C followed by centrifugation for 20 min at 7500 rpm. Collected sera stored at -20 for further analysis.

3.4. Induction of OVA-specific Th2 responses

To induce an allergen specific airway inflammation, Ovalbumin (OVA) was weekly administered as antigen to BALB/c mice, for a period of 4 weeks. For intranasal immunization mice were anaesthetised with intraperitoneal injection of Ketamine/Xylazin (sec. 3.2). After 5-7 min they were lied down onto the back and subjected to intranasal immunization with 50µg Ovalbumin (OVA) (Sigma Grade V, Deisenhofen, Germany) in 50 µl sterile PBS. (Figire 4A). Subsequently, they were challenged intranasally at day 61 and 62 with 50µg OVA/50µg PBS and 24 hours later mice were sacrificed. To obtain a negative control group, instead of OVA, mice were intranasally immunized with PBS (Group - no sensitization), at the same time points of immunization (Figure 4B).

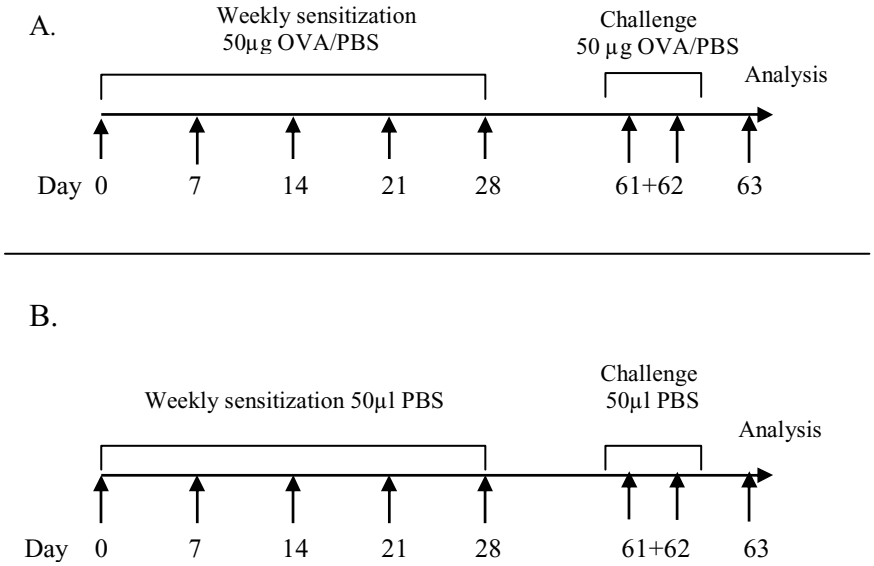


Figure 4. *Immunization scheme*. A. Allergen specific sensitization (7 x OVA), B. no sensitization.

3.5. Experimental setup of allergen specific immunotherapy

After 5 times application of antigen, the allergen sensitization is completed (day 28). Then allergic were mice subjected to allergen specific immunotherapy (SIT), where PBS solutions

with increasing doses of OVA (1µg-1mg) were administered intranasally or alternatively subcutaneously (Figure 5). After completing the allergen specific immunotherapy course, mice challenged twice with 50µg OVA and 24 hours later subjected to analysis. During SIT course, non sensitized and OVA sensitized control groups underwent PBS applications.

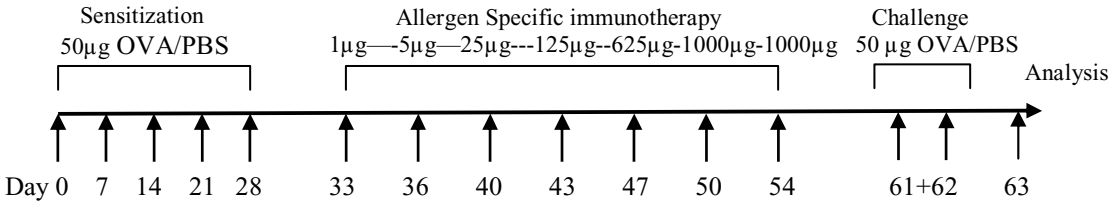


Figure 5. *Allergen immunization and therapy scheme.*

3.6. Production and purification of the IL-4/IL-13 inhibitor

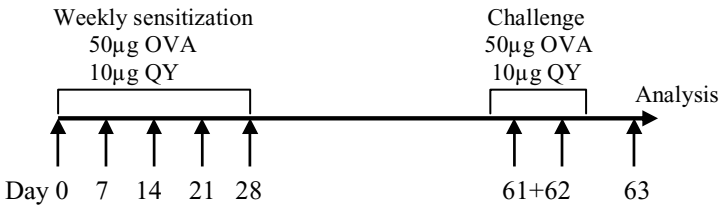
For inhibition of the IL-4/IL-13 system a murine double mutated IL-4 variant was used, which has been constructed previously by site directed mutagenesis (Q116D, Y119D). The cytokine inhibitor was produced in the *E-coli* expression system in a pET-30a vector (Stratagene, Amsterdam, The Netherlands). The protein-containing inclusion bodies were denaturated with 8 mol/L guanidinium chloride, 100mmol/L Tris-HCl pH 8.0, and 2-mercaptoethanol and were renaturated by extensive dialysis against 120mmol/L NaCl, 2mmol/L KCl, 3 mmol/L NaH_2PO_4, and 7 mmol/L Na_2HPO_4. The refolded protein was purified by ionexchange chromatography on a CM-Sepharose Fast Flow column (Pharmacia, Freiburg, Germany) equilibrated with 25 mmol/L ammonium acetate and eluted with a 0 to 0.5 mol/L NaCl gradient. Finally, the material was applied on a reverse phase HPLC column and eluted by a gradient of acetonitrile from 30% to 50%. Remaining endotoxins were removed by affinity chromatography by using AffinityPak Detoxi-Gel column (Pierce/KMF,

St Augustin, Germany) according to the manufacturer´s instructions. Endotoxin concentrations were determined by using Pyrogent Gel-Clot Limulus Amebocyte Lysate Test (Bio Whittaker, Verviers, Belgium) and were less than 0.06 endotoxin units/ml. Final protein concentrations were determined by the bicinchoninic acid method (Pierce/KMF) by using BSA as standard. Before use the inhibitory function of the inhibitor was assessed in vitro by a receptor binding test in the Biacore 2000 System (Biacore, Freiburg, Germany) and inhibition of cell proliferation in the IL-4 sensitive pre B cell line BAF/3. The IL-4 variant was lyophilized and stored at -80°C.

3.7. Application of IL-4/IL-13 inhibitor (QY)

To address the therapeutic potential of IL-4/IL-13 inhibitor in a mouse model for allergic airway inflammation, the inhibitory molecule was tested in established and persisting disease. Allergic sensitization was performed as described above (sec. 3.4). Subsequently, the groups of mice were treated intranasally with 10µg QY/PBS during allergic sensitization – prophylactic regimen (Figure 6A), whereas other groups were therapeutically administered to 10µg QY/PBS, after completion of allergic sensitization (Figure 6B). In addition, IL-4/IL-13 inhibitor was used as an adjuvant for allergen specific immunotherapy, where 10µg QY inhibitor applied intranasally in parallel with increasing doses of OVA (Figure 6C).

A.



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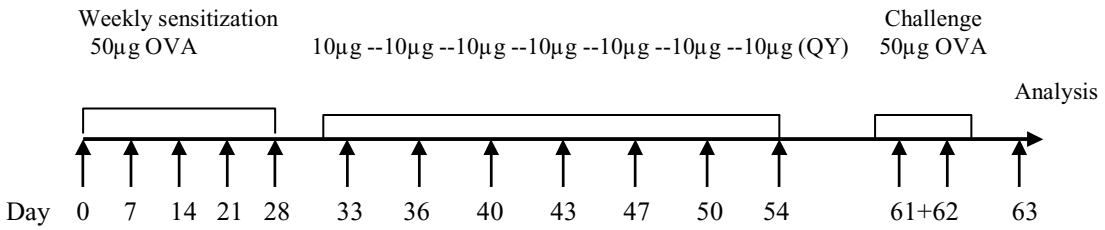


C.

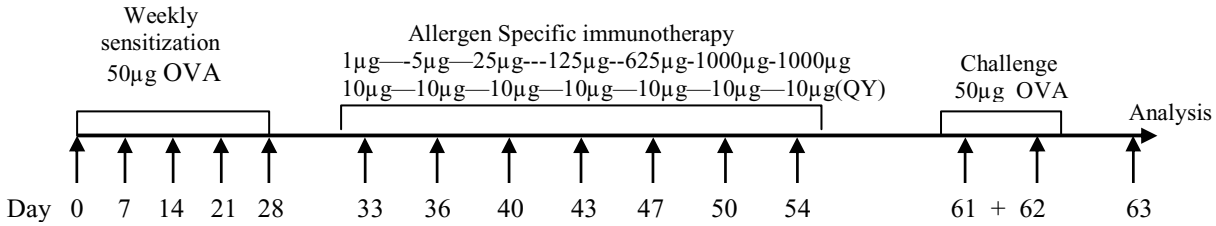


Figure 6. *Application scheme of IL-4/IL-13 inhibitor (QY).* A. Application of QY during allergic sensitization. B. Application of QY after allergic sensitization. C. Combination of QY inhibitor with SIT.

3.8. Application of CD28 superagonist mAb in a mouse model for allergic airway inflammation

To study the therapeutic potential of CD28 superagonist mAb (D665), the previously described mouse model for allergic airway inflammation was used (sec 3.4). Different doses of a-CD28 mAb were applied intraperitoneally at various time points of allergic sensitization (prophylactic regimen) or after completion of allergic sensitization (therapeutic regimen), four days before analysis (day 39)(Table 2). Isotype control antibodies (Mouse IgG1, PFR-01) were used as a control, at the same time points as the a-CD28 was administered. For all experiments the read out was performed 24 h after the last challenge at day 43.

	Sensitization						Challenge
Day	0	7	14	21	28	39	41+42
Negative controls	PBS Iso	PBS	PBS Iso	PBS	PBS Iso	Iso	PBS
	PBS 50µg a-CD28	PBS	PBS	PBS	PBS		PBS
	PBS 100µg a-CD28	PBS	PBS	PBS	PBS		PBS
	PBS 50µg a-CD28	PBS	PBS 50µg a-CD28	PBS	PBS 50µg a-CD28		PBS
Prophylactic treatment	OVA 50µg a-CD28	OVA	OVA	OVA	OVA		OVA
	OVA 100µg a-CD28	OVA	OVA	OVA	OVA		OVA
	OVA 50µg a-CD28	OVA	OVA 50µg a-CD28	OVA	OVA 50mg a-CD28		OVA
Therapeutic treatment	OVA	OVA	OVA	OVA	OVA	50µg a-CD28	OVA
	OVA	OVA	OVA	OVA	OVA	100µg a-CD28	OVA
Positive contol	OVA Iso	OVA	OVA Iso	OVA	OVA Iso	Iso	OVA

Table 2. *Experimental design of administration of a-CD28 mAb´s.*

3.9. Bronchoalveolar Lavage (BAL)

In order to evaluate an allergic airway inflammation, mice were sacrificed. After lethal anaesthesia mice bled from the axillar vein, to reduce further blood contamination with lung tissue during Bronchoalveolar lavage (BAL) preparation. The trachea was cannulated and BAL was performed by flushing lung and airways 3-4 times with 1ml sterile PBS containing 10% fetal calf serum (FCS) (PAA laboratories, Linz, Austria).

3.10. Determination of differential cell counts in the BAL fluid

BAL total cells were counted with a cell counting camera, therefore similar amounts (1:1) of BAL fluids and Trypan Blue solution (Sigma-Aldrich, Steinheim, Germany) were mixed together. Subsequently, BAL fluids were spun onto the glass slides (10 min at 600U/min) using a cytospin (Shandon Southern Products Ltd., Asmoor, UK) and air dried glass slides were stained with Diff-Quick (Dade Behring, Marburg, Germany) according to the manufacturer's instructions. The number of different cell types – eosinophils, neutrophils, macrophages, lymphocytes were determined microscopically by using standard cytological criteria.

3.11. Lung Histology

Lung tissues were fixed in 10 % phosphate-buffered formalin for 24 hours and embedded in paraffin wax. Embedded tissues were cut to 2-3 µm sections followed by staining with Hematoxilin-Eosin (H&E). The stained sections visualized by light microscopy and examined for inflammatory infiltrates.

3.12. Detection of cytokines by ELISA

For the detection of IL-4, IL-5, IFN-γ and IL-10, in the BAL fluid and cell culture supernatants sandwich ELISA's (OptEIA-SET, BD Pharmingen, San Diego, CA) were used. The assays performed in polyvinyl chloride microtiter plates (Dynatech, Denkendorf, Germany) according to the manufacturer's instructions. The plates were coated with unconjugated capture mAb for mouse IL-4, IL-5, IFN-γ or IL-10 overnight at 4°C. Next day the plates were washed and then blocked with assay diluent (10% FCS/PBS). After washing step undiluted BAL fluids or cell culture supernatants and recombinant standards were

applied into the plate wells and incubated for 2h at RT. The detection performed with biotinylated anti-mouse IL-4, IL-5, IFN- γ or IL-10, respectively. The binding reactions visualized with a conjugate of streptavidin-Horseradish peroxidase (HRP) followed by incubation with the substrate TMB reagent (BD Pharmingen, San Diego, CA). After 30 min reaction stopped with 1M phosphoric acid and absorbance was read at 450 nm in an ELISA micro plate reader (SLT Spectra, Tecan, Crailsheim, Germany).

3.13. Measurement of serum OVA specific antibody titers

Circulating OVA specific IgE and IgG subclasses were measured in the sera by ELISA. 96-well plates were coated over night at 4°C with 100µl 0.1 mol/L $NaHCO_3$ containing 100µg OVA/ml. Then plates were blocked for 2 hours at 37°C with 200µL 3% Bovine serum albumin (BSA) in PBS. Plates washed, and 100µl of 8 series with 2-fold serum dilutions in PBS containing 1% BSA was applied overnight at 4°C. The amount of bound antibody was detected using either Biotinylated monoclonal antibodies against mouse IgE (R35-118), followed by visualization with a conjugate of streptavidin-HRP or using horseradish peroxidase conjugated antibodies against mouse heavy chain classes (for the detection OVA specific IgG1 and IgG2a subclasses). After 45 min of incubation at RT with TMB substrate (BD Pharmingen, San Diego, CA) the reaction stopped with 1M phosphoric acid. Plates were read in a microplate autoreader (SLT Spectra, Tecan, Crailsheim, Germany) at 450nm. Quality of ELISA's and detection antibodies was controlled using a laboratory standard serum pool, which was applied on each ELISA plate and was collected 20 days after sensitization with OVA. Data evaluated by determination of serum titers, which was the serum dilution lying 1.5 fold over background optical density of non immunized mice.

3.14. Cell preparation and in vitro T cell activation

Single-cell suspensions from the mediastinal lymph nodes or from spleens were prepared by teasing them through a steel mesh and discarding the cell debris. The cells were counted with cell counting camera in dilution to 1:10 of cell suspension /Trypan Blue solution and resuspended at $2x10^6$ cells/ml in cell culture medium (RPMI 1640 medium supplemented with 10 mM Hepes, 2mM L-Glutamine, 10% FCS, 10µg/ml streptomycin, 10 U/ml penicillin, 50µM 2-mercaptoethanol). The cellular suspensions (200µl) were applied onto a cell culture plates and either left in medium alone, or were cultured with 10µg OVA. Alternatively, the plates were coated over night at 4°C with mAb to CD3ε (145-2C11, 10µg/ml) (BD/Pharmingen, San Diego, CA) and stimulated in the presence of recombinant human IL-2 30µl/well (200U/ml), (Novartis, Basel, CH) at 37°C in a humidified atmospare containing 5% CO_2. After 48h cell culture supernatants were harvested and tested for the presence of cytokines.

3.15. Immunofluorescent staining of intracellular cytokines with Flow Cytometry

Single cell suspensions were prepared from spleens. The cells were resuspended at $2x10^6$ cells/ml in RPMI medium containing 10% FCS and then stimulated with phorbol ester (5µg/ml) and calcium ionophore (0.5 µM) for 4 hours (both reagents from Sigma) at 37°C in a humidified atmosphere containing 5% CO_2. Brefeldin A (2µg/ml, Sigma) was added for the last 2h of the in vitro culture period. The stainings were performed according to the instructions from Pharmingen. Briefly, after 4 hours of stimulation, cells were washed with FACS Buffer (PBS containing 2.5% FCS and 0.1% sodium azide (Merck, Darmstadt, Germany)) and were fixed with 4% formalin in PBS for 20 min at RT and followed by 20 min incubation with anti-CD16/CD32 mAb (2.4G2; Fc Block (5µg/ml)). Subsequently, cells were

stained with FITC-labelled anti-CD4 mAb (L3T4) and PerCP-Cy5.5-labled anti CD25 (PC61) for the detection of Treg cells. After 15 min of staining in the dark, cells were permeabilized 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 and additionally incubated with anti-CD16/CD32 mAb (2.4G2; Fc Block (5µg/ml)). This was followed by intracellular staining of IL-10 with PE labelled anti-IL-10 mAb (JES5-16E3), resuspended in the permeabilization buffer. Finally cells were washed with permeabilization buffer and FACS buffer and analyzed using BD FACS Canto Flow cytometer (Becton Dickinson). Specificity of antibody binding was controlled by staining with isotype matched control antibodies. All antibodies were purchased from Pharmingen.

3.16. Detection of FoxP3 positive cells by flow cytometry

The staining of FoxP3 positive cells was performed using the APC anti-mouse/rat FoxP3 Staining Set (eBioscience, San Diego, USA), according to the manufacturer´s instructions. Briefly, the single cell suspensions were prepared from spleens and resuspended at $1x10^6$ cells/ml. After washing step with Fax buffer, cells were incubated with anti-CD16/CD32 mAb (2.4G2; Fc Block (5µg/ml)) for 15min at 4°C, in the dark, followed by extracellular stainings of CD4 with FITC-labeled anti-CD4 mAb (L3T4) (Pharmingen) and CD25 with PE-labeled anti-CD25 mAb (PC61)(eBioscience). 30min later stained cells were washed and subsequently fixed and permeabilized with Fix/Perm buffer, for 2h at 4°C in the dark. Then, permeabilized cells were additionally stained with anti-CD16/CD32 mAb (2.4G2; Fc Block (5µg/ml)) and without washing step stained for intracellular FoxP3 with APC labelled anti-FoxP3 mAb (FJK-16s), for 30min at 4°C. Finally, washed cells were resuspended with FACS

buffer and analyzed using BD FACS Canto Flow cytometer (Becton Dickinson). Specificity of antibody binding was controlled by staining with isotype matched control antibodies.

3.17. Statistical analyses

Statistical significance for normally distributed samples were analyzed by an unpaired *t*-test. Non-normal data or data with unequal variances were tested for significance using the Mann-Whitney Rank Sum Test. Values of $P<0,05$ were considered statistically significant.

4. RESULTS

4.1. In vivo mouse model for allergic airway inflammation

To study the immune mechanisms of allergen specific immunotherapy, a previously established mouse model for allergic airway inflammation was used (Hahn et al., 2003), which shares many features of asthma in humans. For the establishment of the allergen specific Th2 state, mice are immunized five times intranasally with 50µg Ovalbumin (OVA) (sec. 3.4) and after an allergen free interval they are subsequently challenged two times with 50µg OVA.

Intranasal allergen sensitization resulted in an increased and persisting OVA-specific IgE synthesis in the blood serum (Figure 7) and was associated with an enhanced synthesis of IL-4 in the BAL fluids (Figure 7).

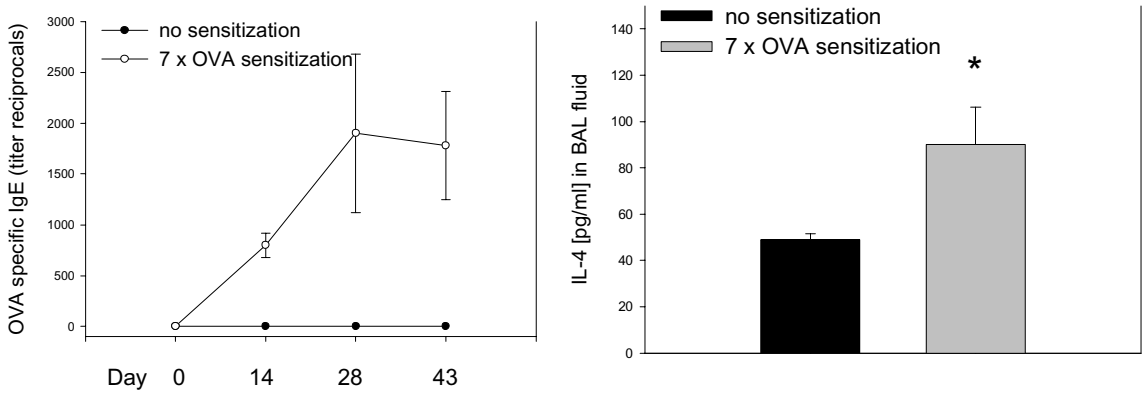


Figure 7. *Serum OVA specific IgE and IL-4 in BAL fluid*. Data are given as means +/- SEM. *P<0,05 vs non sensitized mice. Mann-Whitney rank sum test.

Furthermore, the described mouse model showed an increased eosinophilic infiltrate in the BAL fluid and in H&E stained lung tissue sections, when compared to non sensitized subjects. These findings was correlated with an enhanced IL-5 synthesis in the BAL fluid of OVA sensitized mice (Figure 8).

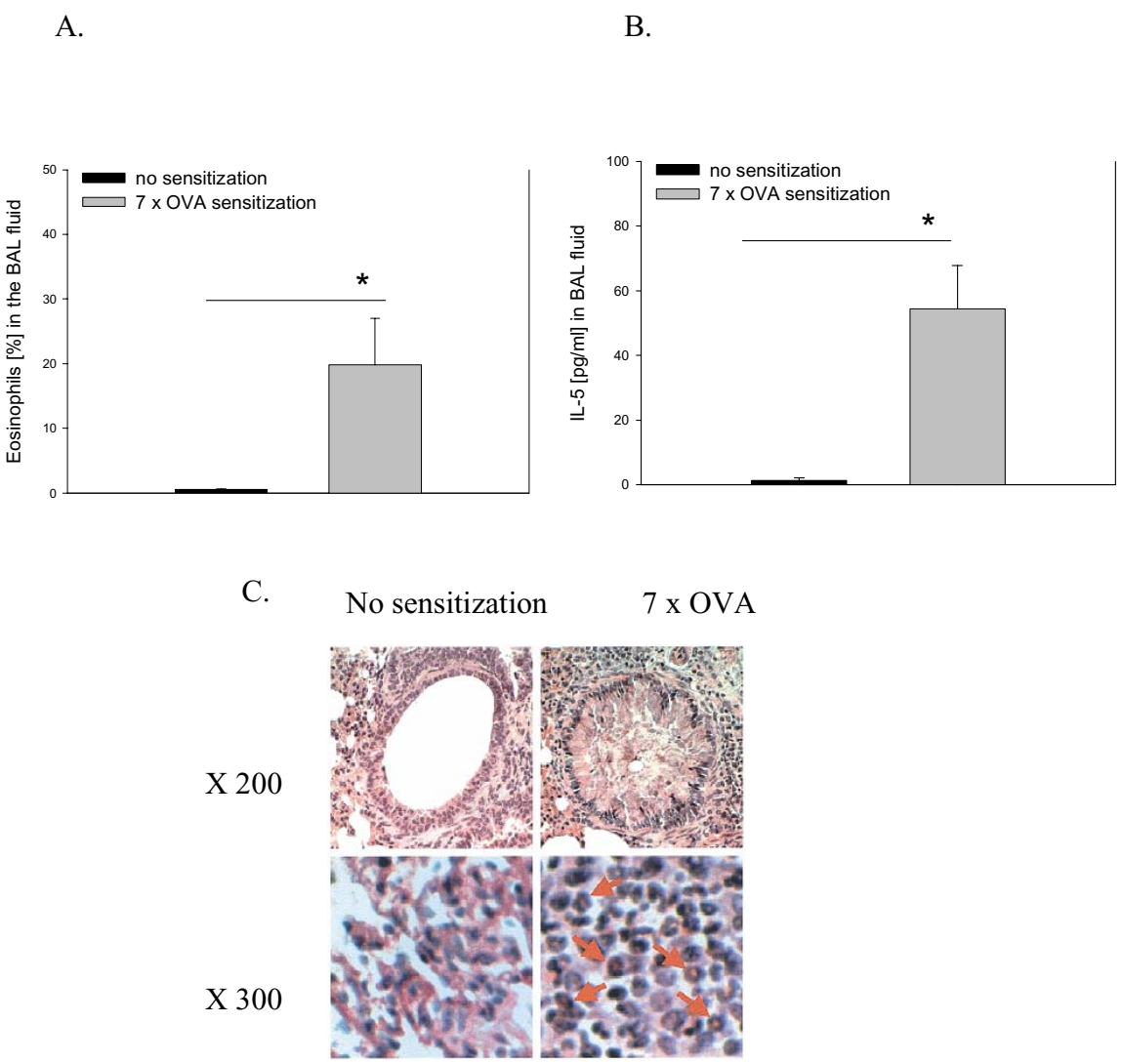


Figure 8. *Allergic airway pathologic condition*. A.Percentage of Eosinophils in BAL fluid; B. IL-5 levels in BAL fluid measured by ELISA. Data are given as means */- SEM, *P<0,05, unpaired *t* test. C. Lung tissue sections were stained with H&E and examined microscopically for inflammatory infiltrate.

All above mentioned findings suggest, that intranasal application of antigen efficiently established an allergen specific Th2 state and approves the use of this model to study various strategies for allergy therapy.

4.2. Establishment of an efficient route of Allergen Specific Immunotherapy in a murine model for allergic airway inflammation

The first goal of the present study was the establishment of allergen specific immunotherapy (SIT) in a mouse model for allergic airway inflammation, that could show similar effects as observed in human studies. In order to efficiently reverse an allergic phenotype in this murine system, two different routes of SIT application were suggested - the traditional subcutaneous and the intranasal administration of increasing doses of specific allergen, directly to the effector organ.

To evaluate the efficiency of different routes of SIT, several independent experiments were performed using the similar experimental design (Table 3).

	Immunization					Allergen specific immunotherapy							Challenge	Analysis
Groups	Day 0	7	14	21	28	33	36	40	43	47	50	54	61+62	63
No sensitization	PBS					PBS							PBS	--
7 x OVA	OVA					PBS							OVA	--
7 x OVA + SITi.n.	OVA					OVA (1µg-1mg) i.n.							OVA	--
7 x OVA + SITs.c.	OVA					OVA (1µg-1mg) s.c.							OVA	--

Table 3. *Experimental design of allergen specific immunotherapy*. OVA-Ovalbumin.

BALB/c mice were weekly immunized and subsequently challenged intranasally with 50µg OVA. 24 hours after the last challenge, animals were sacrificed and several clinical and immunological parameters were analysed. Therapeutic groups were subjected to increasing doses of OVA from 1µg to 1mg intranasally or alternatively subcutaneously, after completion of allergic sensitization.

First, OVA specific antibodies were detected in sera. Figure 9 shows that the treatment of allergic animals with intranasal SIT significantly reduced the OVA specific IgE levels,

whereas the subcutaneous application only slightly reduced synthesis of allergen specific IgE. In parallel with the reduction of OVA specific IgE´s, we observed in both groups significantly increased titers of OVA specific IgG1, in comparison to OVA sensitized untreated mice.

In contrast to the subcutaneously treated groups that show no significant differences regarding to OVA specific IgG2a titer, the intranasal treatment resulted in a remarkable increase of OVA specific IgG2a in comparison to OVA sensitized untreated groups (Figure 9C).

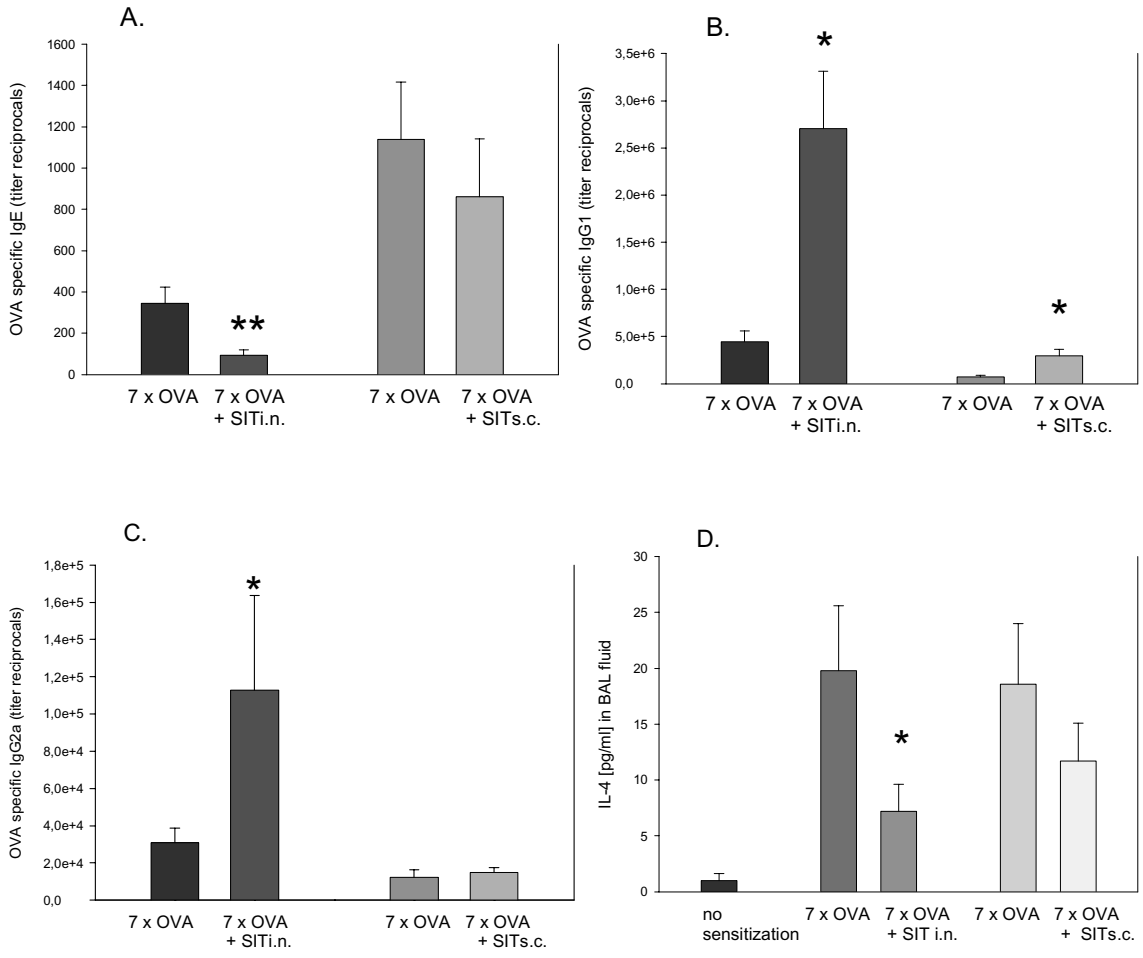


Figure 9. *Serum OVA specific antibodies and IL-4 levels in BAL fluids*. A: IgE, B: IgG1, C: IgG2a, D: IL-4. Titer of OVA specific antibodies in the sera and IL-4 levels in the BAL fluids were determined by ELISA after completion of sensitization and therapy courses. Data are given as means (n=8-10) +/- SEM,. *P<0,05, **P<0,005 vs OVA sensitized untreated mice. Mann-Whitney rank sum test.

The reversal in the immunoglobulin induction was associated with a considerable suppression of IL-4 synthesis in the BAL fluids of intranasally treated mice (Figure 9D). Furthermore, here we demonstrate that subcutaneous administration of SIT also reduces the IL-4 levels in the airways, when compared to OVA sensitized untreated mice, however this suppressive effect was statistically not significant. Moreover, the analysis of IL-4 secretion in *in vitro* stimulated spleen cell culture supernatants either with a-CD3/IL-2 or specifically with OVA, did not show any significant trends between the untreated and the SIT treated groups (data not shown).

Subsequently, the differential cell counts in the lavage fuids were determined, using standard cytological criteria.

	Groups				
Cells 10^4/ml	no sensitization	7 x OVA	7 x OVA + SITi.n.	7 x OVA	7 x OVA + SITs.c.
Total cells	11±2,1	12,4±2,6	10,2±1,2	8,25±1,0	10,4±2,3
Eosinophils	0,1±0,03	2,6±0,8	0,5±0,2**	2,3±0,8	1,1±0,4
Neutophils	1,7±0,2	3,2±0,5	3,0±0,6	1,5±0,2	2,8±0,5*
Lymphocytes	0,1±0,03	0,3±0,06	0,25±0,05	0,45±0,2	0,4±0,09
Macrophages	11,3±1,9	7,4±1,0	6,4±0,8	3,8±0,7	5,7±0,9

Table 4. *Absolute numbers of total cells and the different cell types present in the airways*. Data are given as means (n=8-10) +/- SEM. *P<0,05, **P<0,005 vs OVA sensitized untreated mice, Mann-Whitney rank sum test.

As shown in Table 4, none of the therapeutic routes had the influence on the total cell numbers in the airways, in comparison to non-sensitized or OVA sensitized untreated groups. However further observation of stained BAL cells revealed considerable difference between the groups. As it is already described (sec. 4.1), sensitization of mice with OVA results in the strong airway inflammation indicated by the increase of eosinophils in the BAL fluid.

Subsequently, the treatment of allergic mice with allergen specific immunotherapy remarkably suppressed the eosinophil number in the airways.

In the present study, the comparison of different routes of SIT administration clearly demonstrate, that the direct application of SIT to the effector organ reduces the airway inflammation more efficiently, than the subcutaneous regimen, where we see only a slight reduction of the eosinophilic infiltrate in the airways (Table 4).

In order to confirm the clinical efficiency of SIT in this experimental model for allergic airway inflammation, IL-5 was detected in the BAL fluids, which is the responsible cytokine for differentiation and maturation of eosinophils. Interestingly, a direct relation between the eosinophil numbers and IL-5 levels in the BAL fluids was found. As figure 10 demonstrates a significant suppression of IL-5 was detectable in the BAL´s of intranasally treated mice which correlates with a remarkable reduction of airway eosinophilia in these subjects.

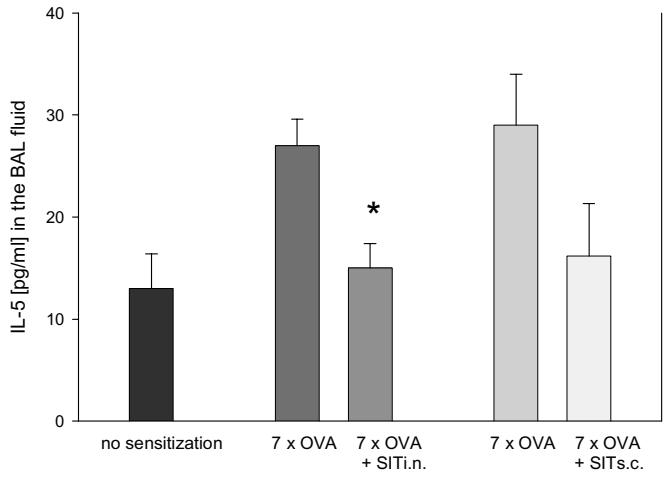


Figure 10. *IL-5 levels in the BAL fluids*. IL-5 levels were measured by ELISA after completion of the sensitization and therapy courses. Data are given as means (n=9-10) +/- SEM. **P<0,005 vs OVA sensitized untreated mice, unpaired *t* test.

Although, the subcutaneous administration of increasing doses of specific antigen during allergen specific immunotherapy reduced the production of IL-5 and decreases eosinophilic infiltrates in the BAL fluids, these effects were not significantly different when compared to OVA sensitized untreated groups. These findings are in line with other tested parameters such as OVA specific antibody titers and IL-4 levels.

In the present study, we wanted to investigate whether the allergen specific immunotherapy is able to reverse the allergic phenotype due to the modulation of Th2/Th1 responses, as it was described by several authors (Secrist et al., 1993)(Jutel et al., 1995)(Ebner et al., 1997). On the other hand we wanted to check whether the Th2/Th1 shift is the central mechanism or a secondary effect of successful immunotherapy. Since in the past few years, it has been proposed that the induction of IL-10 producing regulatory T-cells is one of the key event of successful SIT in patients (Till et al, 2004)(Akdis et al., 1998).

To test both hypotheses in our SIT model, first the IFN-γ levels were measured in the BAL fluids and in *in vitro* stimulated spleen cell culture supernatants. No significant differences in the levels of IFN-γ were found in the BAL fluids as well as in cell culture supernatants, stimulated either with a-CD3/IL-2 or with OVA, from different groups of mice (data not shown).

Subsequently, we checked whether the synthesis of IL-10 secretion is affected through the application of SIT to allergic mice. Figure 11 illustrates the levels of IL-10 in BAL fluids, measured by ELISA.

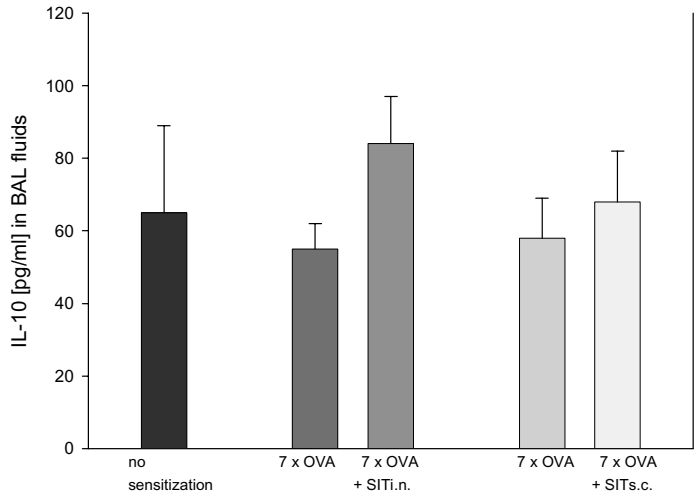


Figure 11. *IL-10 levels in BAL fluids*. IL-10 levels were measured by ELISA after completion of the sensitization and therapy courses. Data are given as means (n=9-10) +/- SEM. **P<0,005 vs OVA sensitized untreated mice, unpaired *t* test.

Here we show, that OVA sensitization resulted in the suppression of IL-10 production, when compared to non sensitized mice. Furthermore, we observed that intranasal therapy restores this reduction significantly, that was not the case in subcutaneously treated animals.

Taking together these findings indicate that the described mouse model for allergic airway inflammation is a suitable system to study different therapeutic interventions in allergy. Moreover, based on the obtained results we demonstrated that the intranasal immunotherapy is efficiently reversed the allergic phenotype in a similar way as in human studies, in contrast to the subcutaneous regimen that only showed little effects in this murine system.

4.3. IL-4/IL-13 inhibitor as adjuvant for allergen specific immunotherapy

After the establishment of intranasal allergen specific immunotherapy in our mouse model, we wanted to investigate the therapeutic potential of an IL-4/IL-13 inhibitor as adjuvant for allergen specific immunotherapy. We used the already described murine system of allergic

airway inflammation and subsequently treated mice with intranasal SIT alone or in combination with the QY IL-4/IL-13 inhibitor. Furthermore, to demonstrate the anti-allergic effect of QY inhibitor, groups of mice were integrated that received cytokine immunotherapy during or after allergic sensitization as indicated in Table 5.

	Sensitization	Therapy	Challenge	Analysis
Day	0 7 14 21 28	33 36 40 43 47 50 54	61+62	63
7 x PBS	PBS	PBS	PBS	
7 x OVA	OVA	PBS	OVA	
7 x OVA +SIT	OVA	1µg-1mg OVA	OVA	
7 x OVA +SIT/QY	OVA	1µg-1mg OVA 10µg QY	OVA	
7 x OVA + QY(after sens.)	OVA	10µg QY	OVA	
7 x OVA + QY(during sens.)	OVA 10µg QY	PBS	OVA 10µg QY	

Table 5. Sensitization and therapy scheme.

Mice were bled during the experiments and sera were checked for OVA specific antibodies. IgE titres were measured by ELISA. Data were collected in 3 independent animal experiments. Data shown are from one representative experiment.

Intranasal OVA sensitization resulted in increased IgE synthesis at day 32 after allergic sensitization was completed and persisted at the end of the experiment. Similar to human SIT, intranasal SIT in mice led to a steady and significant reduction of OVA specific IgE synthesis at the end of the experiment. IgE levels in mice treated with SIT in combination with the QY IL-4/IL-13 inhibitor, showed similar levels as mice treated with allergen immunotherapy

alone (Figure 12). Mice, however, which had received an immunotherapy with the IL-4/IL-13 inhibitor alone after allergic sensitization was completed, did not show significant decreases of IgE titers, although mean values were decreased when compared to untreated OVA sensitized mice. As a control for the efficacy of the IL-4/IL-13 inhibitor, mice were treated prophylactically during allergen sensitization. As demonstrated previously (Hahn C. et al., 2003), these mice showed a significant reduction of OVA specific antibodies, when compared to untreated OVA sensitized mice.

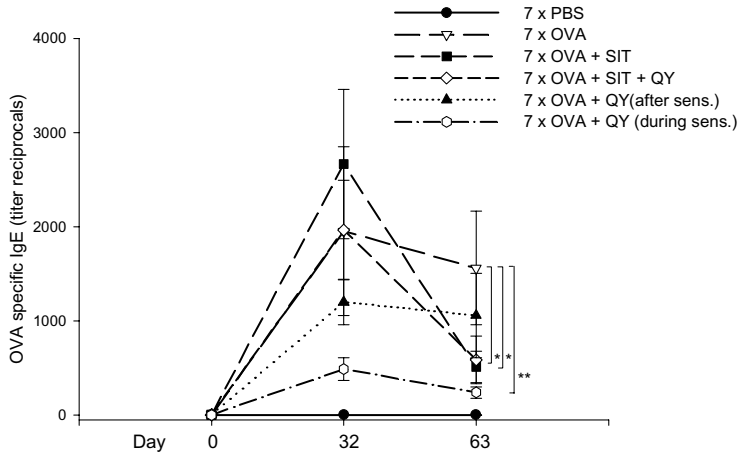


Figure 12. *OVA specific IgE´s.* After sensitization and therapy courses IgE levels were measured by ELISA. Data are given as means (n=7-8) +/- SEM. **P .001, Mann-Whitney rank sum test.

Next we analyzed sera for OVA specific IgG1 and IgG2a antibodies. Both immunoglobulin isotypes increased under intranasal SIT when compared to untreated OVA sensitized mice. Animals, which have been treated with SIT in combination with the QY IL-4/IL-13 inhibitor showed no significant difference in comparison to mice treated with SIT alone. In contrast, as published previously (Grunewald SM. et al., 1998) both treatment with the QY inhibitor during allergic sensitization, as well after allergic sensitization suppressed specific IgG1 and

IgG2a antibody synthesis when compared to untreated OVA sensitized mice (Figure 13).

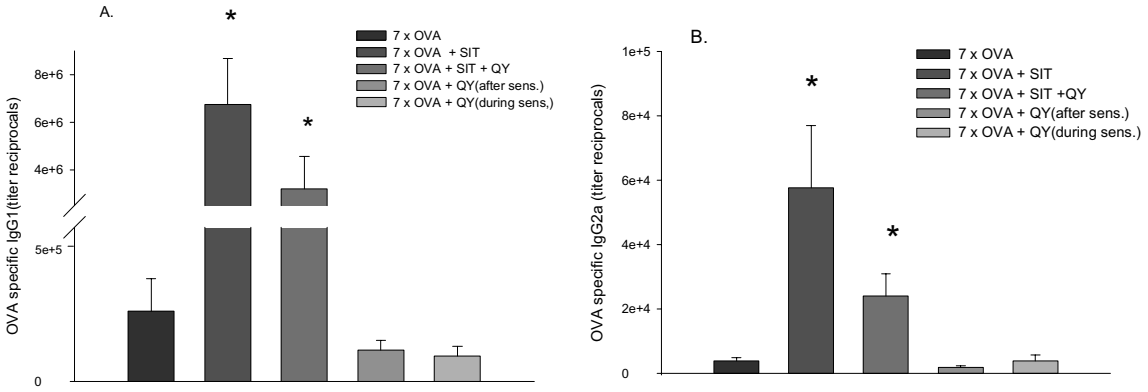


Figure 13 *OVA specific IgG´s*. After sensitization and therapy courses Ig levels were measured by ELISA. Data are given as means +/- SEM. **P .001, Mann-Whitney rank sum test. (n=7-8). A. OVA specific IgG1, B. OVA specific IgG2a.

After sensitization and therapy mice were challenged twice intranasally. Mice were sacrificed 24 hours later and bronchoalveolar lavages were performed. All groups showed similar total cell counts in the BAL fluid (data not shown). Intranasal OVA sensitization, however, resulted in increase of eosinophils in the bronchoalveolar lavage fluid in comparison to non sensitized mice (Figure 14).

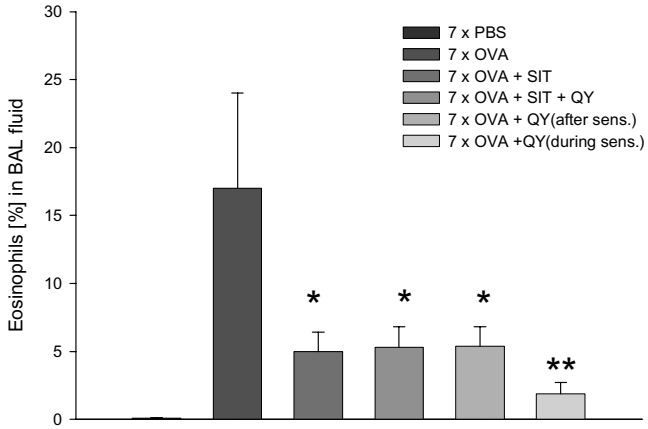


Figure 14. *Airway eosinophilia*. After completion of the sensitization and therapy courses, BAL´s were performed and the amount of eosinophils was determined. Data are means (n=7-8) +/- SEM , *P .05, **P .001, unpaired t-test. (n=5-7).

Mice, which received intranasal SIT showed a significant reduction of eosinophils in the bronchoalveolar lavage fluid, when compared to untreated OVA sensitized mice. Cytokine directed immunotherapy alone after allergic sensitization also resulted in a significant reduction of the eosinophil amounts. The combination of allergen specific and cytokine directed immunotherapy, however, did not induce a further decrease of eosinophil amounts in the BAL fluid (Figure 14), when compared to mice treated with SIT alone or cytokine inhibitor alone in each of the 3 independent animal experiments performed. As demonstrated previously (Hahn C. et al., 2003), the prophylactic application of the QY inhibitor during allergic sensitization resulted in a complete inhibition of airway eosinophilia in comparison to untreated OVA-sensitized mice.

The histological analysis of lung sections showed similar results (Figure 15). Allergic sensitization was associated with a strong peribronchial eosinophil rich inflammatory infiltrate, which was decreased in mice treated with SIT alone. The combination of SIT and immunotherapy with the QY cytokine inhibitor, however did not show any obvious differences in comparison to mice, which received only SIT. In line with the decreased eosinophilia in the BAL fluid, the inhibition of IL-4/IL-13 system during allergic sensitization resulted in decreased peribronchial eosinophilic infiltrates in the lung tissues, which confirms the efficiency of QY inhibitor in this mouse system.

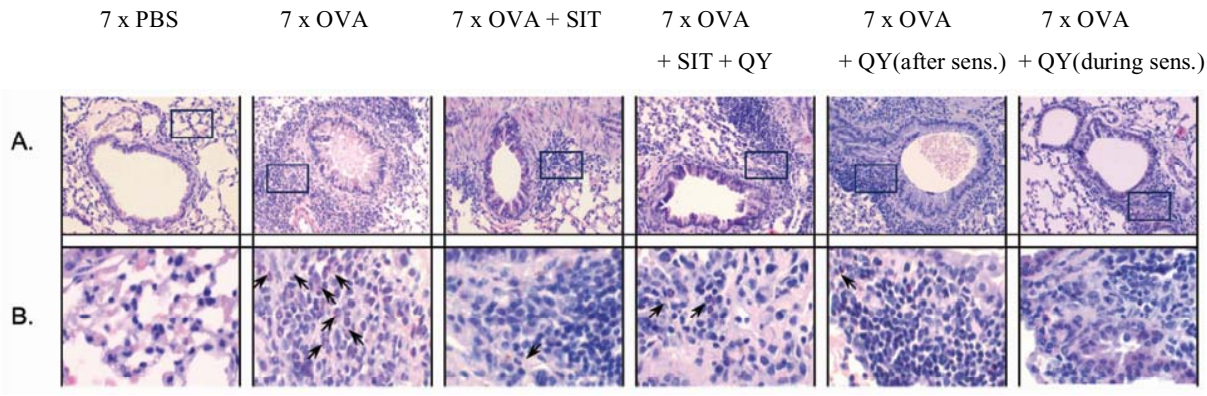


Figure 15. *Lung histologies*. Lung tissues were fixed and embedded in paraffin wax. Sections were stained with Hematoxylin-Eosin (H&E). Magnification A: 200x, B: 300x.

Next we investigated the cytokine profiles in the BAL fluids as well as in spleen cell suspensions after *in vitro* restimulation with anti CD3/IL-2 or specifically with OVA.

In congruence with the eosinophils in the BAL fluid, IL-5 levels were significantly increased in OVA sensitized mice, when compared to non sensitized control group. Mice, which have been subjected to SIT showed a significant reduction of IL-5 levels, when compared to OVA sensitized group. In mice, which were treated with SIT in combination with the QY IL-4/IL-13 inhibitor, there was no significant difference of IL-5 values in the BAL fluid in comparison to mice treated with SIT alone (Figure 16). Group of mice, which were treated after allergic sensitization with QY inhibitor instead of SIT, showed decreased mean IL-5 levels in the BAL fluid, when compared to untreated OVA sensitized mice. However, this difference was not significant. The inhibition of the IL-4/IL-13 system during allergic sensitization, however, reduced IL-5 levels significantly, when compared to untreated OVA sensitized group (Figure 16). In vitro restimulated spleen cell cultures did not show any significant differences in IL-5 levels between the groups (Figure 16). Moreover, in OVA stimulated spleen cell cultures IL-5 had a non detectable level, similar to the cell suspensions taken from the unstimulated medium cultured cells.

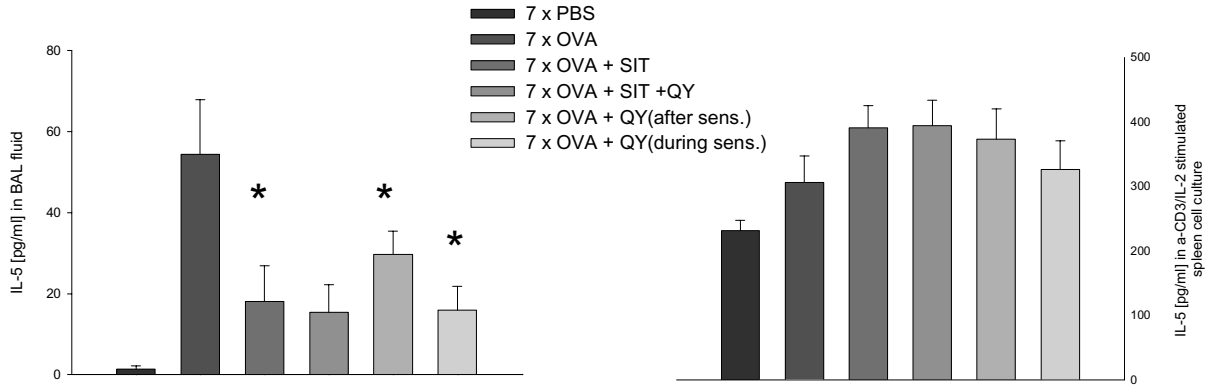


Figure 16. IL-5 levels were measured in the BAL fluids and in vitro a-CD3/IL-2 stimulated spleen cell cultures by ELISA after completion of the sensitization and therapy courses. Data are means (n=7-8) +/- SEM. *P .05, **P .001, unpaired t-test.

Furthermore IL-4 levels in the bronchoalveolar lavage fluid were found to be increased in OVA sensitized mice in comparison to non sensitized control animals. Allergen immunotherapy resulted in decrease of IL-4 levels, when compared to OVA sensitized mice. Secretion of IL-4 in the BAL fluid was elevated in mice, which were treated both with the QY IL-4/IL-13 inhibitor alone and in combination with SIT, when compared to mice treated with SIT alone. Furthermore, mice, which were treated prophylactically and subsequently challenged with the QY inhibitor, showed highest levels of IL-4 in BAL fluid. This reflects the cross reactivity of the ELISA detection antibody between the wild type and the QY IL-4/IL-13 inhibitor, which is a mutated IL-4 molecule, and indicates remaining amounts in the lung of the intranasally applied inhibitory protein (Figure 16). However, in mice that were subjected to IL-4/IL-13 inhibitor during allergic sensitization this effect was restored by decreased levels of IL-4 in a-CD3/IL-2 stimulated spleen cell cultures. Analysis of IL-4 secretion in a-CD3/IL-2 as well as OVA stimulated spleen cell cultures, show no significant trends between the other groups. Similar to IL-5 levels, in OVA stimulated or in unstimulated medium cultured spleen cell supernatants, we could not measure detectable levels of IL-4.

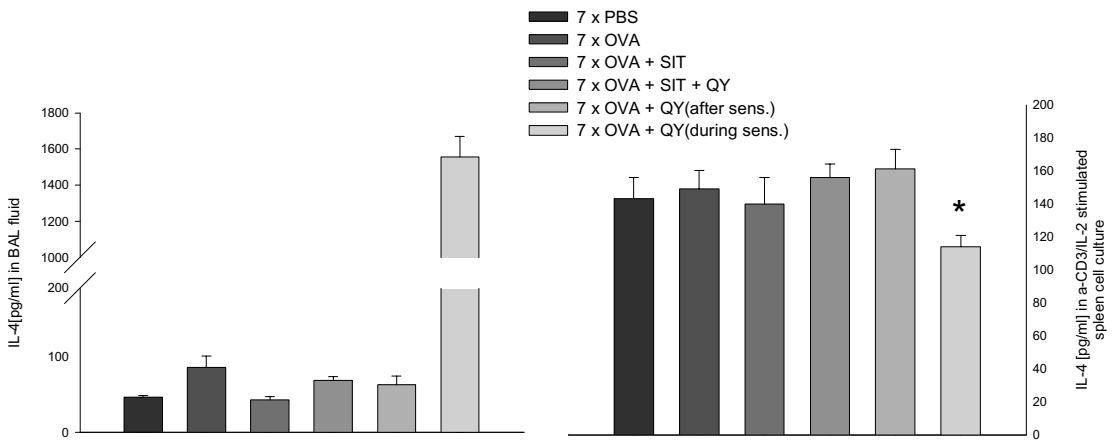


Figure 17. IL-4 levels were measured in the BAL fluids and in vitro a-CD3/IL-2 stimulated spleen cell cultures by ELISA after completion of the sensitization and therapy courses. Data are means (n=7-8) +/- SEM. *P .05, **P .001, unpaired t-test

In addition IL-13 levels were determined in the BAL fluid as well as after *in vitro* T cell stimulation with anti CD3/IL-2. IL-13 levels were found to be at baseline levels of non sensitized mice showing no significant differences in all groups (data not shown).

The IFN-γ production was determined in BAL fluids and in *vitro* stimulated spleen cell cultures, however no significant differences were found between the groups (Figure 18).

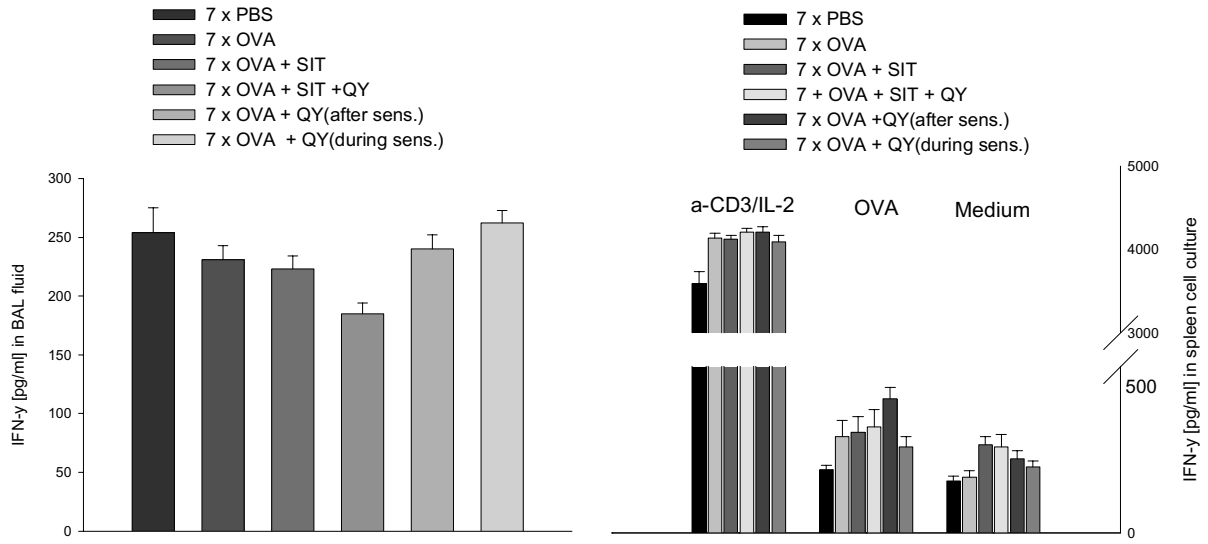


Figure 18. *IFN-y levels were measured in the BAL fluids and in vitro stimulated spleen cell cultures by ELISA after completion of the sensitization and therapy courses*. Data are means (n=7-8) +/- SEM. *P .05, **P .001, unpaired t-test

In addition, we measured IL-10 levels in the BAL fluids and in *in vitro* stimulated spleen cell cultures, in order to detect the induction of an IL-10 producing regulatory T-cell response. OVA sensitization decreased IL-10 levels in the BAL fluid in comparison to non sensitized mice. Allergen specific immunotherapy restored IL-10 amounts in the BAL back to levels of non sensitized mice. Mice, which received SIT in combination with the QY IL-4/IL-13 inhibitor showed similar increased IL-10 levels in the BAL as mice, which were treated with SIT alone. IL-10 levels in the BAL fluid of mice, which were treated with QY cytokine inhibitor both during and after allergic sensitization were at baseline levels of non sensitized animals (Figure 19).

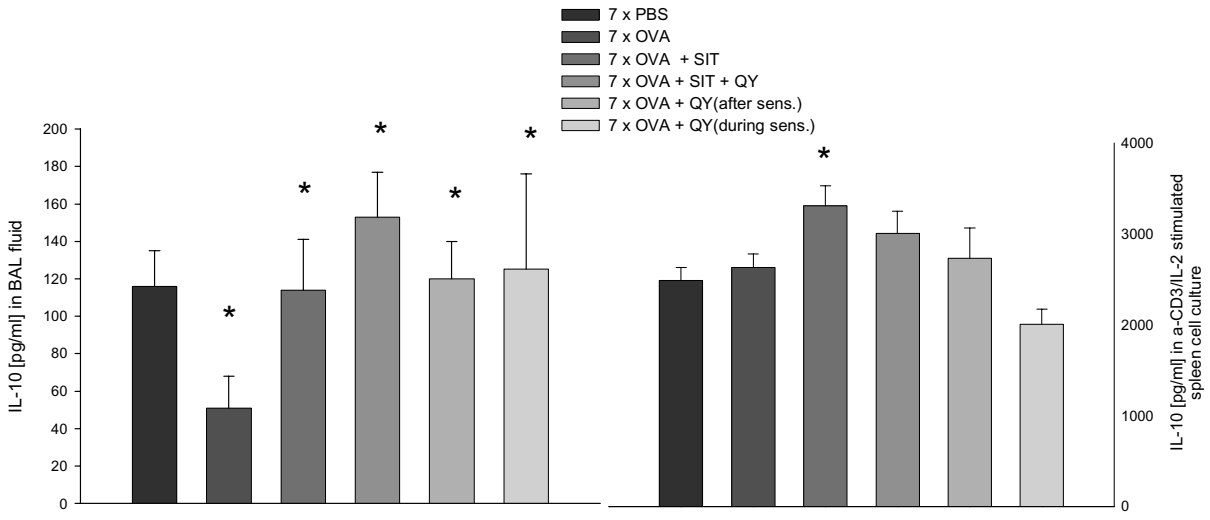


Figure 19. *IL-10 levels were measured in the BAL fluids and in vitro stimulated spleen cell cultures by ELISA after completion of the sensitization and therapy courses*. Data are means (n=7-8) +/- SEM. *P .05, **P .001, unpaired t-test.

An a-CD3/IL-2 stimulated spleen cells supernatants showed increased IL-10 levels in mice, that were treated with SIT when compared to untreated OVA sensitized mice and non sensitized control mice. However, SIT in combination with IL-4/IL-13 inhibitor, did not show

any significant differences in comparison to mice treated with SIT alone. In OVA or in medium cultured spleen cell supernatants there was undetectable level of IL-10.

Next, we wanted to know, whether the above mentioned increase of IL-10 in the BAL fluid and in restimulated spleen cell suspensions was associated with an increased percentage of IL-10/CD25 positive regulatory T cells. Therefore, spleen cells were stained for CD4 and CD25, and subsequently for intracellular IL-10. Stained cells were analysed by flow cytometry.

Indeed, we could show that the number of IL-10 positive CD25 positive CD4 T cells was significantly increased in mice, which received SIT (0,44 +/- 0,02, n=7) when compared to untreated OVA sensitized mice (0,3 +/- 0,04, n=7, p=0,002) and non sensitized mice (0,28 +/- 0,03, n=8). However, no significant differences were found between mice, which received SIT in combination with the QY cytokine inhibitor (0,34 +/- 0,02, n=8) and mice which received the QY inhibitor during allergic sensitization (0,25 +/- 0,03, n=7) or after allergic sensitization instead of SIT (0,33 +/- 0,02, n=7) when compared to untreated OVA sensitized mice.

Thus, taking together our results indicate, that intranasal allergen specific immunotherapy efficiently reverse the allergic phenotype by reducing specific IgE synthesis and airway eosinophilia. This was associated with decrease of IL-4, IL-5 synthesis in BAL fluids and increased IL-10 levels in BAL´s and spleen cell cultures. In addition, FACS staining revealed the association between the amount of IL-10 and increased percentage of IL-10 secreting CD4+CD25+ T cell population. However, mice treated with IL-4/IL-13 inhibitor during SIT, did not show any significant differences.

4.4. The role of regulatory T cells in allergy

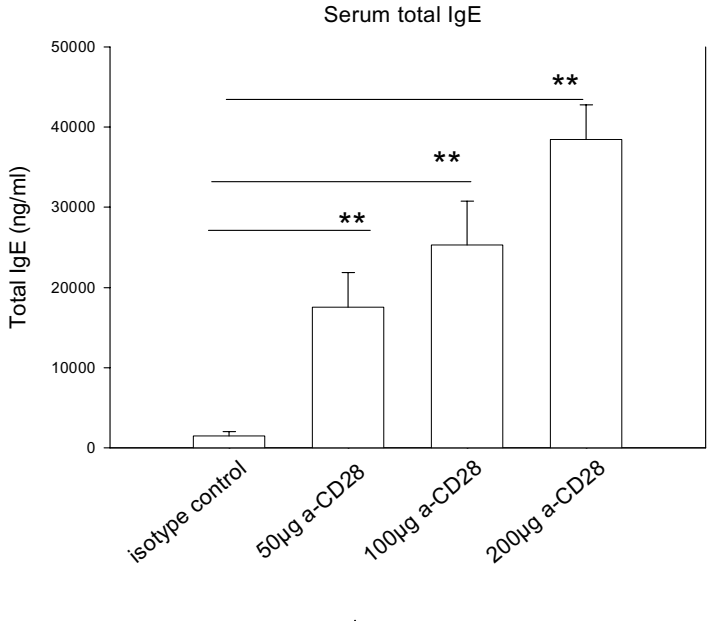
4.4.1. The prophylactic application of a CD28 superagonist in a mouse model for allergic airway inflammation

During last couple of years there is an increasing evidence that the development of allergic disease such as rhinitis, asthma and atopic eczema is controlled by several populations of regulatory T cells including CD4+CD25+ Treg (Umetsu et al.,2003). With regard to these T cell subsets, recently demonstrated that *in vivo* administration of CD28 superagonist leads to the preferential expansion and strong activation of naturally occurring CD4+CD25+CTLA-4+FoxP3+ Treg cells over conventional T cells (Beyersdorf N., 2005)

The following study was designed in order to investigate the effects of CD28 superagonist mAb in our murine system. Therefore first we performed a precursor experiment, where the groups of non sensitised mice were treated with various doses (50µg, 100µg, 200µg) of a-CD28 or with isotype control antibodies as a negative control group. After 7 days total serum IgE and various cytokines in *in vitro* restimulated spleen cell cultures were measured by ELISA. Application of a-CD28 to non sensitized mice induced a dose dependent increase of total serum IgE and increased the secretion of the cytokines IL-4, IL-10 and IFN-y (Figure 20) in a-CD3/IL-2 stimulated spleen cell cultures, when compared to the negative control group.

Furthermore, the intracellular staining of IL-10 in CD4+CD25+ T cells, revealed the dose dependent increase of these T cell populations as already demonstrated previously (Lin and Hünig, 2003).

A.



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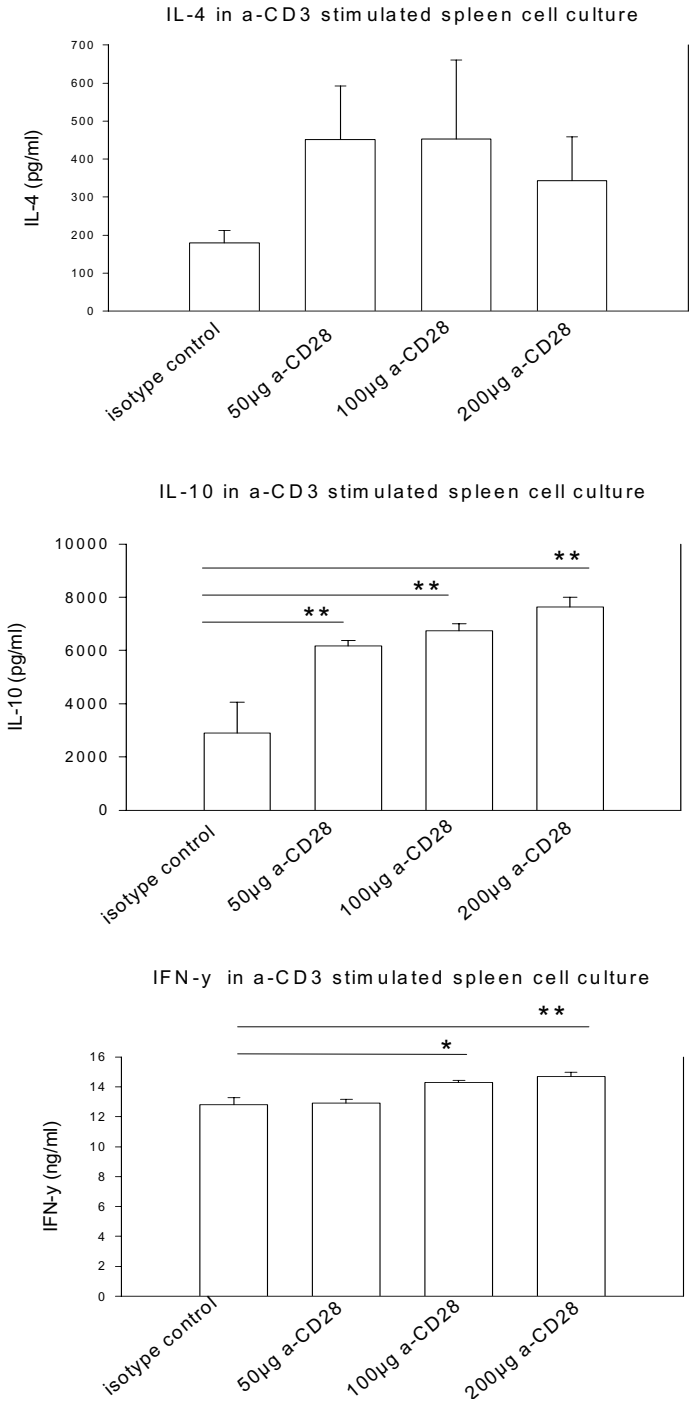


Figure 20. *Serum total IgE and cytokines in in vitro a-CD3/IL-2 stimulated spleen cell culture*. A: Total IgE, B: IL-4, IL-10 and IFN-γ levels measured by ELISA. Data are given as means (n=3) +/- SEM,. *P<0,05, **P<0,005 vs isotype control group, unpaired t test.

Next, we performed a second animal experiment (Table 6) where various doses of a-CD28 (Gr. 3,5,7) or an isotype control antibody (Gr.10) were applied during allergic sensitization. Read out was performed 24 h after the last allergen challenge at day 43.

		Sensitization	Therapy	Number of animals
Negative controls:	Group 1:	7 x PBS	4 x 100µg Iso (day 0, 14, 28 39)	3
	Group 2:	7 x PBS	1 x 50µg a-CD28 (day 0)	5
	Group 4:	7 x PBS	1 x 100µg a-CD28 (day 0)	5
	Group 6:	7 x PBS	3 x 50µg a-CD28 (day 0, 14, 28)	5
Positive control:	Group 10:	7 x OVA	4 x 100µg Iso (day 0, 14, 28, 39)	5
Prophylactic treatment:	Group 3:	7 x OVA	1 x 50µg a-CD28 (day 0)	5
	Group 5:	7 x OVA	1 x 100µg a-CD28 (day 0)	5
	Group 7:	7 x OVA	3 x 50µg a-CD28 (day 0, 14, 28)	5
Therapeutic treatment:	Group 8:	7 x OVA	1 x 50µg a-CD28 (day 39)	5
	Group 9:	7 x OVA	1 x 100µg a-CD28 (day 39)	5

Table 6**.** *Experimental set up of a-CD28 application.*

Whereas the OVA specific IgE`s where not detectable in non sensitized a-CD28 treated mice (Gr. 2,4,6), the treatment with a-CD28 together with OVA sensitization resulted in a dose dependent increase of serum OVA specific IgE´s (Figure 21).

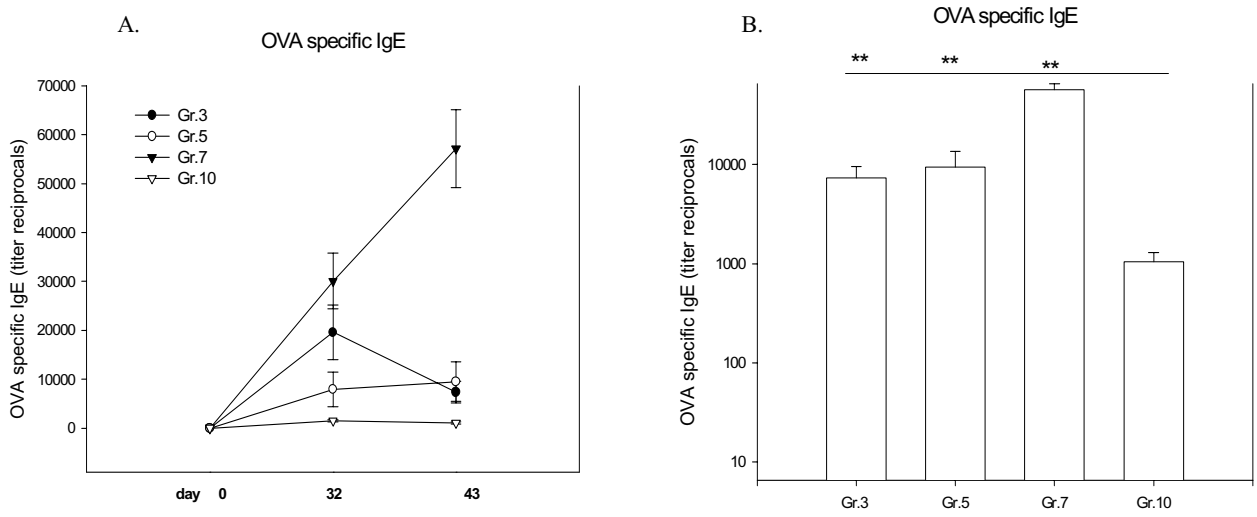
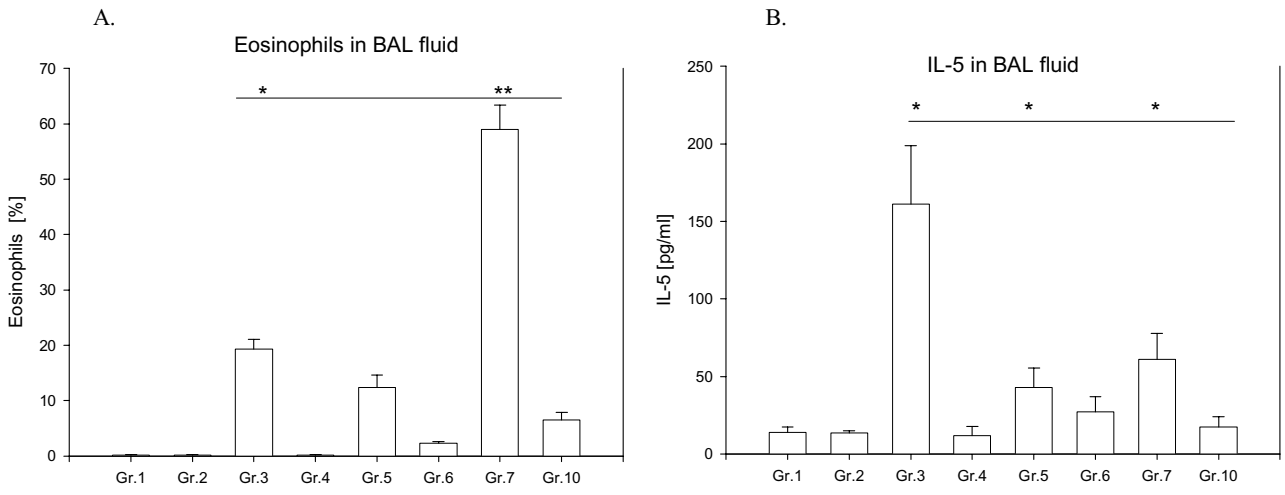


Figure 21. *Titers of* serum *OVA specific IgE.* A. Dynamic of OVA specific IgE at different time points of experiment, B. OVA specific IgE at day 43. Titers of OVA specific antibodies determined by ELISA. Data are given as means (n=5) +/- SEM,. *P<0,05, **P<0,005 vs positive contol group (Gr.10), Mann-Whitney rank sum test.

Furthermore the amount of Eosinophils in the BAL fluid was increased in OVA sensitized a-CD28 treated mice, when compared to OVA sensitised mice, which were subjected to the isotype control. The percentage of Eosinophils was extremely high in group 7, which received the highest dose of a-CD28. In contrast, non sensitized mice which were treated with the a-CD28 antibody showed no airway eosinophilia (Figure 22). All groups, which received the allergic sensitisation and a-CD28 therapy (groups 3,5,7) showed enhanced levels of IL-5 in the bronchoalveolar lavage (BAL) fluid in comparison to OVA sensitised mice, which were treated with the isotype control (Gr.10)(Figure 22). Herewith, in OVA sensitized a-CD28 treated mice (Gr. 3,5,7) IL-5 levels in the BAL´s were significantly higher than in the non sensitized control groups (Figure 22). In both a-CD3/IL-2 and OVA stimulated spleen cell cultures, OVA sensitized a-CD28 treated mice showed a stronger secretion of IL-5 than OVA sensitized mice, which were treated with isotype control (Figure 22). However, there was no additional IL-5 secretion in OVA sensitized a-CD28 treated animals, when compared to the non-sensitized a-CD28 treated control groups.



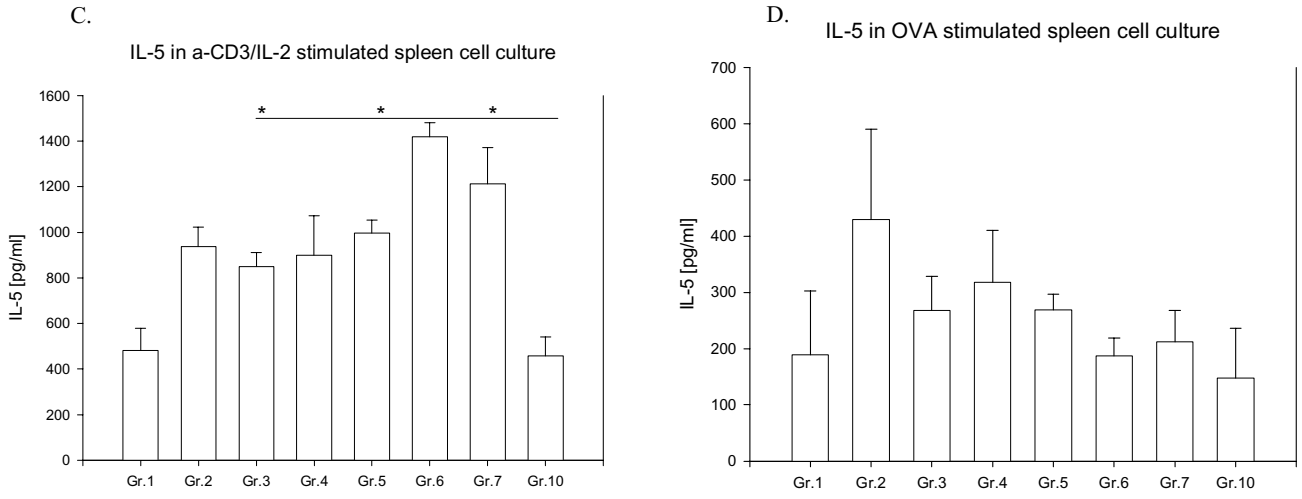


Figure 22. *Percentage of Eosinopils and IL-5 levels.* A. Eosinophils [%] in BAL fluids, B. IL-5 level in BAL fluids, C. IL-5 levels in *in vitro* a-CD3/IL-2 stimulated spleen cell culture, D. IL-5 levels in *in vitro* OVA stimulated spleen cell culture. Data are given as means (n=3-5) +/- SEM, *P<0,05, **P<0,005 vs positive control group (Gr.10), unpaired *t test*.

We did not see significant differences regarding to the IL-4 levels in the BAL fluids between all the groups. The exception was the group, which received 1x50µg a-CD28 at day 0 (Gr.3)(Figure 23). In a-CD3/IL-2 or OVA stimulated spleen cell cultures IL-4 secretion was significantly increased in non sensitized or OVA sensitized animals that received the highest doses of a-CD28 (Gr. 6 and 7), when compared to OVA sensitized control group (Figure 23).

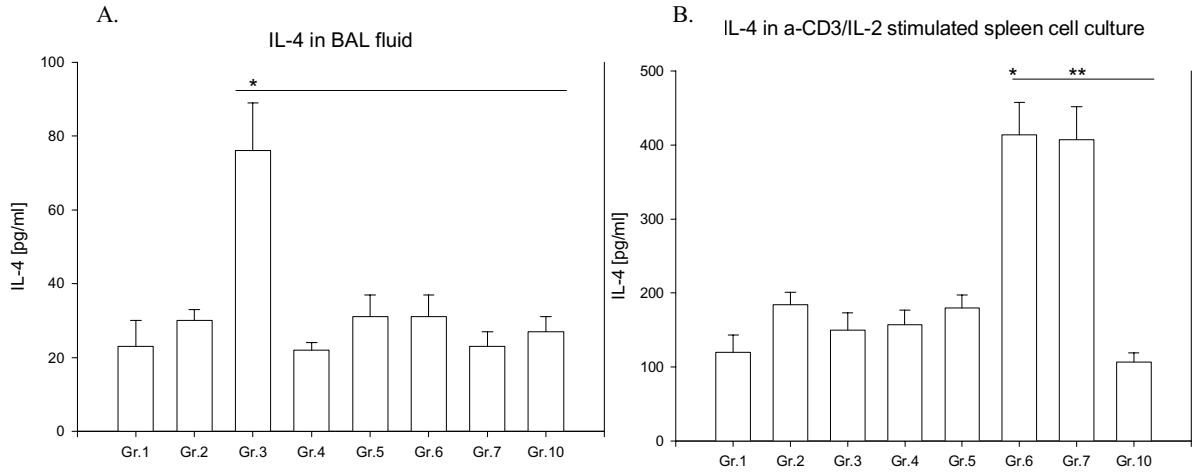
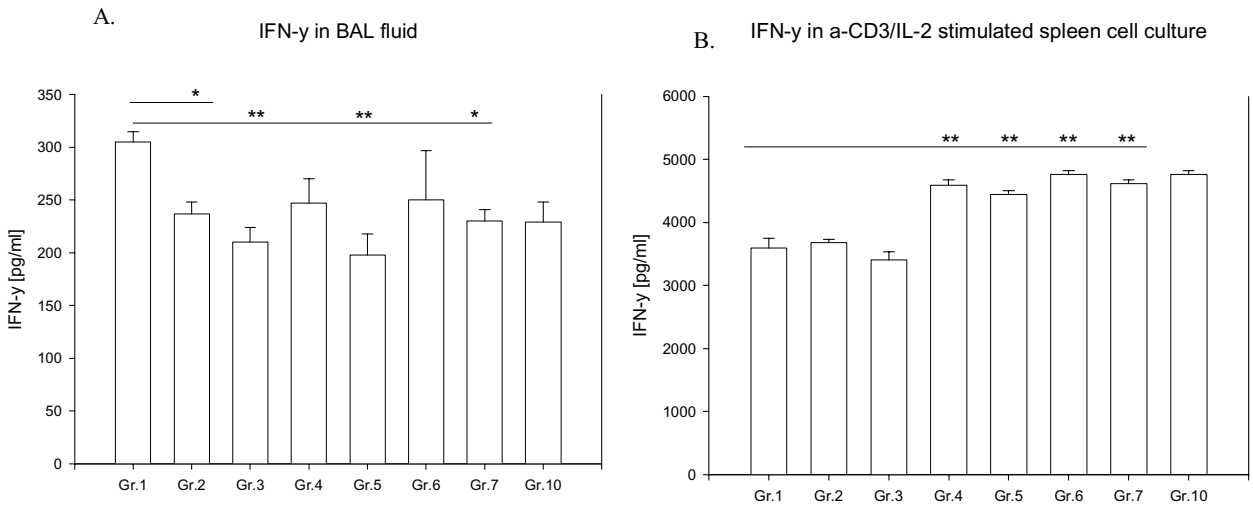


Figure 23. *IL-4 levels measured by ELISA.* A. IL-4 levels in BAL fluids. B. IL-4 levels in *in vitro* a-CD3/IL-2 stimulated spleen cell culture. Data are given as means (n=3-5) +/- SEM,. *P<0,05, **P<0,005 vs positive control group (Gr.10), Mann-Whitney rank sum test.

Subsequently, we measured the IFN-γ levels in the BAL fluid and in spleen cell cultures. Data indicated that the application of a-CD28 to non sensitized mice reduced IFN-γ levels in the BAL fluid, when compared to non sensitised mice, which received the isotype control (Gr.1). This could be further decreased in the groups, which received the allergic sensitization together with a-CD28 antibody therapy (Figure 24). In contrast, we found a dose dependent increase of IFN-γ in a-CD3/IL-2 stimulated cell cultures of non sensitized a-CD28 treated groups (similar results were obtained in the precursor experiment), which was similar in OVA sensitized a-CD28 treated mice. However, after OVA specific stimulation of spleen cells IFN-γ levels were reduced in OVA sensitized a-CD28 treated mice, when compared to non sensitized a-CD28 treated control mice. In OVA sensitized mice, which received the highest dose of a-CD28 (Gr.7) IFN-γ levels were significantly reduced, when compared to OVA treated mice which received the isotype control (Figure 24).



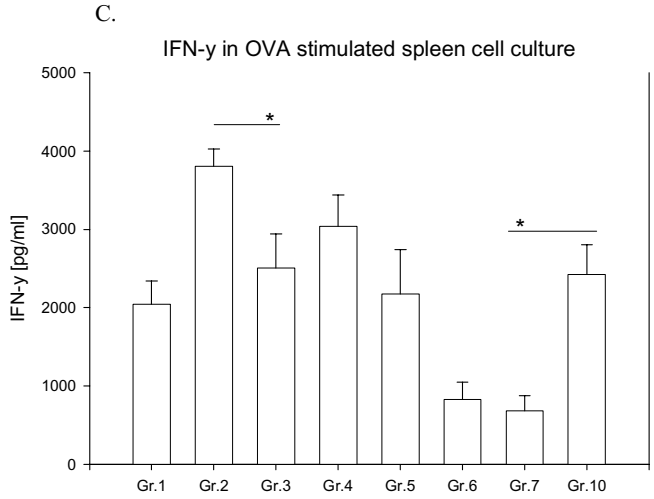


Figure 24. *IFN-γ levels detected with ELISA.* A. IFN-γ in BAL fluids, B. IFN-γ in *in vitro* a-CD3/IL-2 stimulated spleen cell culture, C. IFN-γ in *in vitro* OVA stimulated spleen cell culture. Data are given as means (n=3-5) +/- SEM, *P<0,05, **P<0,005, unpaired *t test*.

IL-10 levels in the BAL fluid seemed to be reduced by allergic sensitisation and was lowest in OVA sensitized mice, which received the isotype control antibody. Treatment with a-CD28 increased IL-10 levels in the BAL fluid in all groups when compared to OVA sensitized mice treated with the isotype control (Figure 25). However, a-CD28 therapy did not significantly increase IL-10 levels in all groups over background levels of non sensitized mice, which received the isotype control (Gr.1). In a-CD3/IL-2 stimulated spleen cell cultures, similar trends could be observed (Figure 25). In OVA stimulated spleen cell cultures, however, no significant differences were found (data not shown).

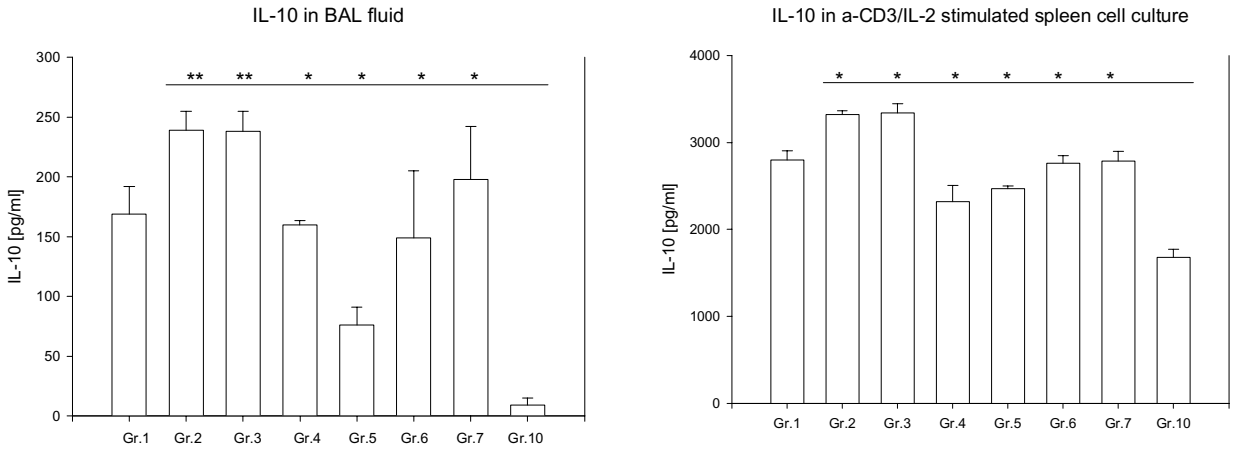


Figure 25**.** *IL-10 levels in BAL fluids and in vitro a-CD3/IL-2 stimulated spleen cell cultures.* Data are given as means (n=3-5) +/- SEM,. *P<0,05, **P<0,005 vs positive control group, Mann-Whitney rank sum test (A.), unpaired *t test* (B).

To test, whether the application of the superagonistic mAb induced T reg cell expansion in our mouse system, we made an extracellular stainings of spleen cells for CD4/CD25 and intracellular stainings for the Treg cell markers FoxP3 and IL-10.

The results indicated that the application of a-CD28 mAb to non-sensitized mice (Gr.2,4,6) induced a dose dependent expansion of CD25+IL-10+ cells among CD4+ T cell population in comparison to non-sensitized/isotype control mice (Gr.1)(Figure 26). In OVA sensitized a-CD28 treated groups these T cell subsets were not further increased (Gr.3,5,7), when compared to non sensitised a-CD28 treated mice. Herewith, the only OVA sensitized group that received the highest dose of a-CD28 (Gr.7) showed a significant increase of CD25+IL-10+ T cell subsets, when compared to positive control group (Figure 26).

Furthermore the FoxP3+ T cell population was analysed within the groups. Surprisingly, no significant trends were observed in all groups (data not shown).

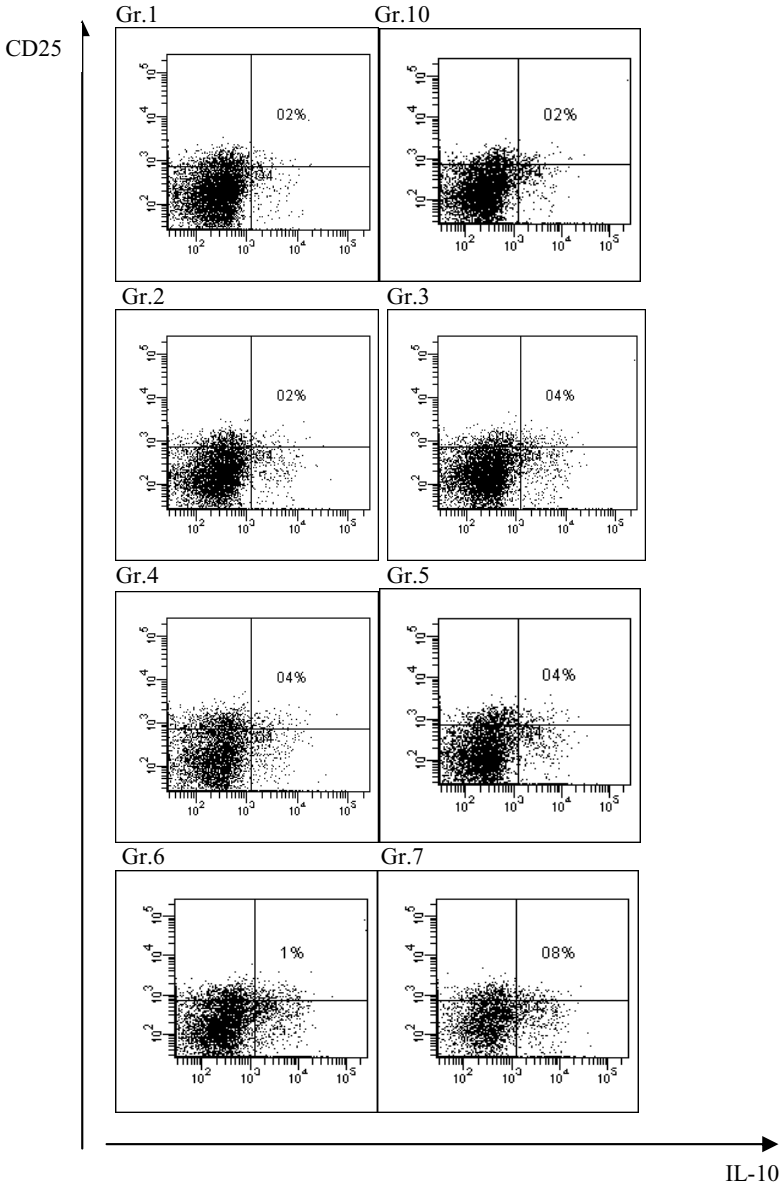


Figure 26**.** *CD25+ IL-10+ T cells among CD4+ spleen T cell subsets. In vitro* activated spleen cells were stained for CD4 and CD25 and subsequently intracellulary for IL-10. Stained cells were measured by FACS. Data are the mean percentage of CD25+ IL-10+ (n=3-5) T cell subsets.

Taken together our results indicate that the application of a-CD28 by itself leads to a dose dependent expansion of CD4+CD25+IL-10+ T cell subsets. Furthermore it induces Th2 polarization with IL-4 and IL-5 cytokine production, which results in the increase of total IgE. The treatment with a-CD28 during allergic sensitization preferably induced Th2 polarization, which could not be downregulated by Treg cells. Despite a slight effects of a single

application of 50µg or 100µg anti CD28 during allergic sensitisation a multiple injections of a-CD28 mAb has stronger influence on all measured parameters.

4.4.2. The therapeutic application of a CD28 superagonist in a mouse model for allergic airway inflammation

In order to investigate the therapeutic effects of a-CD28, the administration of various doses of mAb´s were performed after completion of the sensitization course, four days before analysis (Table 6). Mice, that were treated with 50 µg mAb´s (Gr.8), showed a decrease of OVA specific IgE titers and the application of 100µg (Gr.9) resulted in a significant reduction of this parameter (Figure 27).

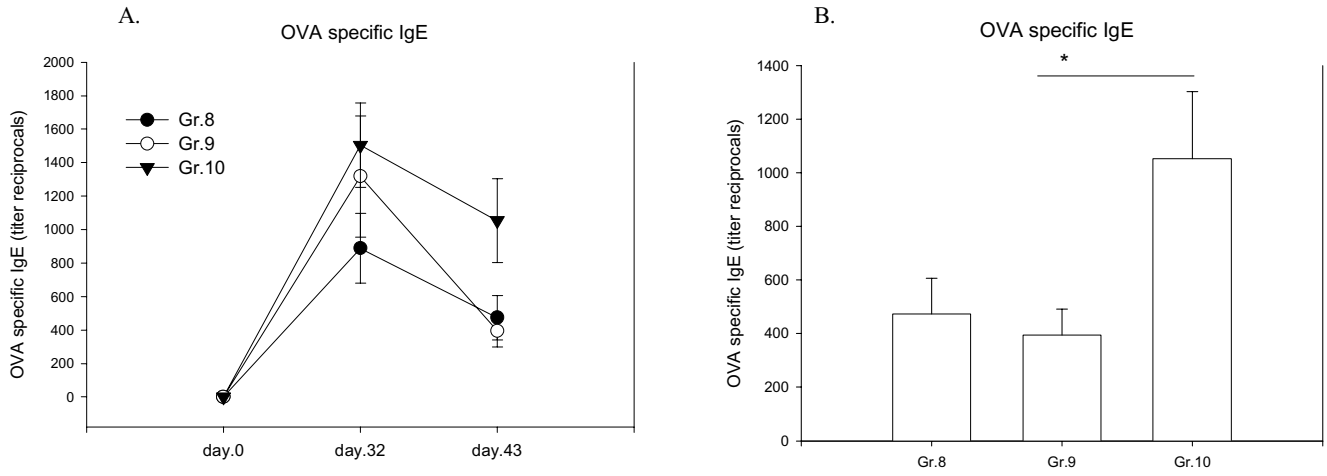


Figure 27. *Titer of* serum *OVA specific IgE.* A. Dynamic of OVA specific IgE at different time points of experiment, B. OVA specific IgE at day 43. Titer of OVA specific antibodies in the sera determined by ELISA. Data are given as means (n=5) +/- SEM,. *P<0,05, **P<0,005 vs positive control group, Mann-Whitney rank sum test.

Furthermore, both groups showed a significantly reduced percentage of airway eosinophils in comparison to positive control group (Gr.8: 1,6±0.4%, p=0,02; Gr.9: 1±0.5%, p=0,006).

Moreover, in the group that received 100µg of a-CD28, IL-4 and IL-5 levels were reduced in the BAL fluid and were nearly completely suppressed after *in vitro* restimulation of spleen cell cultures with OVA in comparison to OVA sensitized mice, which were treated with the isotype control antibody (Figure 28).

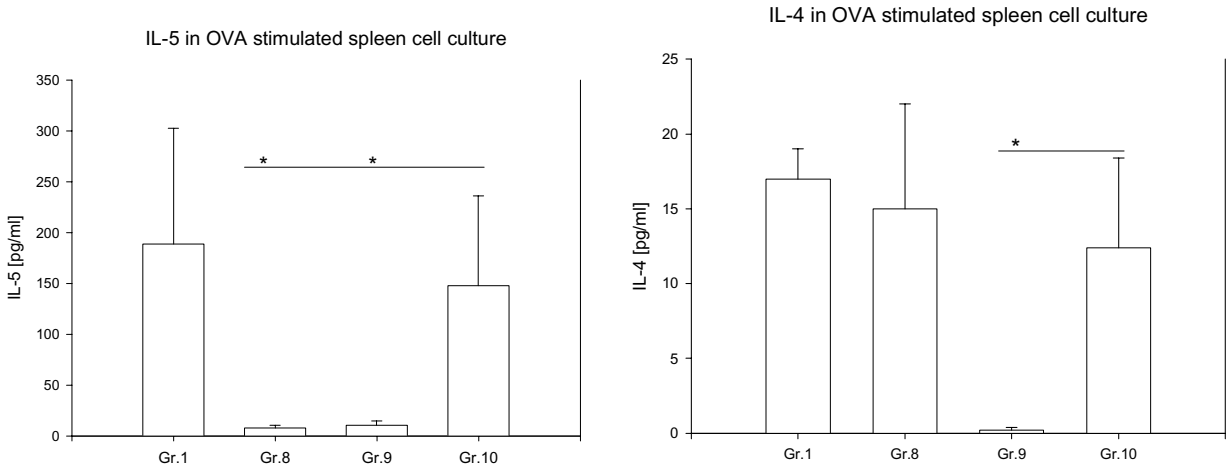


Figure 28. *IL-4 and IL-5 levels in in vitro OVA stimulated spleen cell cultures, detected by ELISA.* Data are given as means (n=5) +/- SEM,. *P<0,05, **P<0,005 vs positive control group, Mann-Whitney rank sum test.

We also analyzed the BAL fluid for secretion of the Th1 cytokine IFN-y, which was significantly increased in both therapeutically treated groups in comparison to OVA sensitised mice, which received the isotype control (Figure 29).

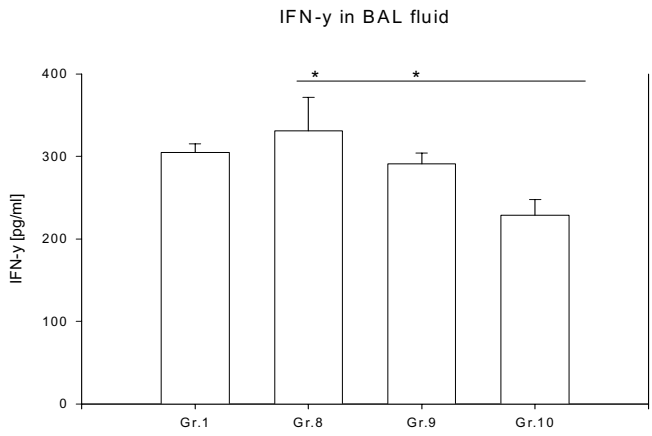


Figure 29. *IFN-γ levels in BAL fluids.* Data are given as means (n=3-5) +/- SEM *P<0,05 vs positive control group, unpaired *t test*.

Furthermore, we detected IL-10 amounts in the BAL fluid and in spleen cell cultures. Our results indicate, that the therapeutic application of mAb to CD28, like in the prophylactic setting, significantly increased IL-10 secretion in the BAL fluid as well as in a-CD3/IL-2 stimulated cell cultures in comparison to OVA sensitized mice, which were treated with the isotype control (Figure 30).

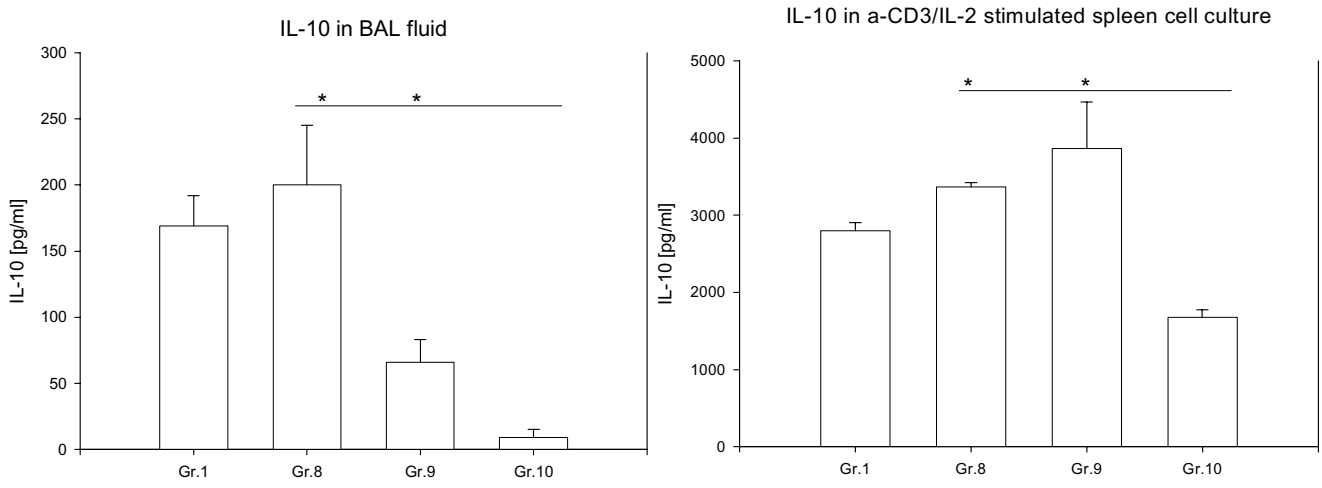


Figure 30. IL-10 levels in BAL fluids and in vitro a-CD3/IL-2 stimulated spleen cell cultures. Data are given as means (n=3-5) +/- SEM P<0,05 vs positive control group, unpaired t test.

In addition, we wanted to know whether the improvement of the allergic state in the therapeutically treated groups is due to the expansion of the T reg cell populations. Therefore we performed FACS analysis, where spleen cells were stained extracellullary for CD4 and CD25 and intracellulary for FoxP3 and IL-10. We observed the expansion of CD25+ IL-10+ secreting cell subsets among CD4+T cell populations in both therapeutically treated groups, which is in line with the IL-10 data (Figure 30). Furthermore, the therapeutic application of a-

CD28 mAb resulted in a significant increase of FoxP3 expression in CD4+CD25+ T cell populations, when compared to negative groups (Figure 31).

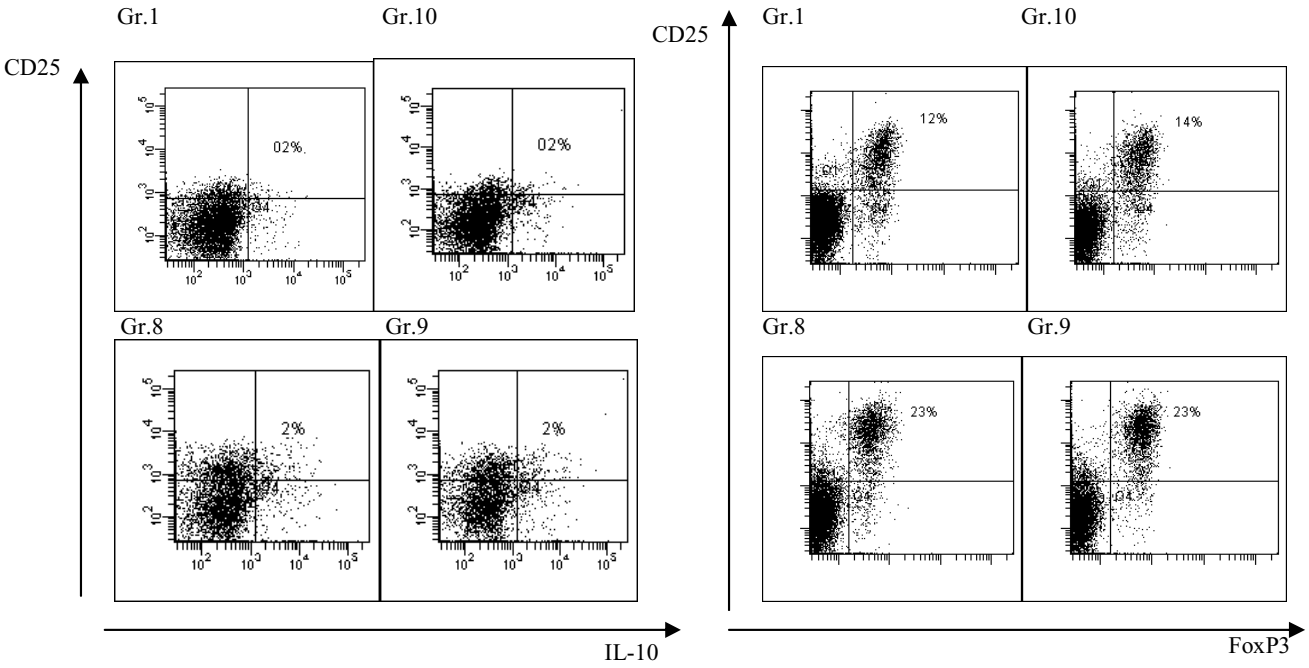


Figure 31. *CD25+ IL-10+ and CD25+ FoxP3+ T cells among CD4+ spleen T cell subsets.* For the staining of intracellular IL-10, the spleen cells were in vitro activated and stained for CD4 and CD25, followed by staining of IL-10. For intracellular detection of FoxP3, non activated spleen cells were stained for CD4/CD25 with subsequent staining of FoxP3. Stained cells were analysed by FACS. Data are given as mean percentages of CD25+IL-10+ or CD25+FoxP3+ (n=3-5) CD4+T cell subsets.

Obtained results indicate that the therapeutic application of a superagonistic mAb to CD28 after allergic sensitization, relieves the allergic condition by reduction of OVA specific IgE and airway eosinophilia in line with the reduction of Th2 cytokines in the BAL fluid as well as in OVA stimulated spleen cell cultures in our mouse system. This is accompanied by an increase of CD25+Foxp3+ and CD25+IL-10+ CD4+T cell subsets. These findings underlie the importance of further investigations to estimate the therapeutic potential of superagonist mAb in allergy therapy.

5. DISCUSSION

5.1. Allergen Specific Immunotherapy in the management of allergic disorders

This thesis work deals with the regulatory mechanisms of allergic airway disorders in a murine system. Since, the allergen specific immunotherapy currently remains as a cornerstone in the management of respiratory allergies, we addressed the present study to the investigation of a mechanism, which leads into the efficiency of this approach. Despite its use in clinical practice for nearly a century, the underlying immunologic mechanisms are slowly being elucidated (Akdis CA. et al., 2004)(Till SJ. et al., 2004).Understanding the immune mechanisms that prevent disease occurrence in nonallergic individuals and studies investigating the regulatory mechanisms under efficient therapeutic approaches in allergic diseases offer promise for new immune interventions.

In our study we established a model for local intranasal immunotherapy. We found, that local intranasal allergen immunotherapy could efficiently reverse the allergic phenotype by reducing OVA specific IgE synthesis and airway eosinophilia significantly in comparison to untreated OVA sensitized animals. Van Oosterhout et al. were the first to publish a mouse model for allergen specific immunotherapy where subcutaneous allergen immunotherapy with OVA efficiently inhibited airway eosinophilia and airway hyperresponsiveness (Van Oosterhout AJ. Et al., 1998) . We demonstrate here, that also local immunotherapy efficiently reversed the allergic phenotype in our murine allergy model. We used a previously established murine asthma model, where intranasal OVA sensitization induces persisting IgE synthesis and an eosinophil rich allergic airway inflammation (Hahn C. et al., 2003). We found, that local intranasal allergen immunotherapy could efficiently reverse the allergic phenotype by significantly reducing OVA specific IgE synthesis and airway eosinophilia in

comparison to untreated OVA sensitized animals. Furthermore, local SIT decreased IL-4 and IL-5 and increased IL-10 levels as well as the amount of IL-10 producing T reg cells. Previous studies addressing the mechanisms of successful SIT reported that SIT induces a shift from Th2 towards a Th1 response (Secrist H. et al., 1993)(Jutel M. et al., 1995). Therefore, we speculated that an IL-4/IL-13 inhibitor as adjuvant during SIT should enhance this effect and improve treatment efficacy. This hypothesis, however, was disproved by our experiments, since mice, which were treated with the IL-4/IL-13 inhibitor during SIT did not show any additional beneficial therapeutic effects when compared to mice treated with allergen immunotherapy alone.

Furthermore, mice were treated prophylactically with the QY IL-4/IL-13 inhibitor during allergic sensitization in our study, in order to underline the antiallergic potential of the inhibitor, which has been demonstrated previously (Hahn C. et al., 2003). Mice, which received the inhibitor during allergic sensitization, showed a significant reduction of OVA specific antibodies. Mice, however, which had received the IL-4/IL-13 inhibitor after allergic sensitization did not show significant decreases of IgE titers. This data is in congruence with the study of Hahn et al., where the QY IL-4/IL-13 inhibitor efficiently prevented the development of an allergic phenotype. In established allergy, however, IL-4/IL-13 inhibition did not significantly ameliorate allergic airway inflammation. In another study it has been described that upon prolonged allergen exposure IL-4 receptor alpha chain knockout mice produced allergen specific IgE`s leading to anaphylaxis (Grunewald SM. et al., 2001). Here we report that IL-4/IL-13 inhibition as adjuvant for allergen immunotherapy does not show additional beneficial effects.

In summary, all these findings together indicate, that although IL-4 and IL-13 are major players for inducing an allergic immune response, other factors seem to control an established

allergy, where IL-4 and IL-13 play a minor role. One candidate cytokine might be IL-5, which is essential for the maturation, differentiation and survival of eosinophils in allergic responses (Lewis DB., 2002). Recent works has shown the potential importance of another Th2 cytokine, IL-9. IL-9 has been shown to act on many cell types involved in asthma, including T-cells, B-cells, mast cells, eosinophils and epithelial cells. The development of transgenic mice over expressing IL-9 has suggested an important role in the development of the asthmatic phenotype, including eosinophilic inflammation, bronchial hyperresponsiveness, elevated IgE-levels and increased mucus secretion (Temann UA. et al., 1998)(Soussi-Gounni A. et al., 2001).

The dose and application mode of the IL-4/IL-13 inhibitor might also be substantial for treatment effects. An efficient resorption and the *in vivo* inhibitory activity of the intranasally applied IL-4/IL-13 inhibitor is indicated by the fact, that prophylactic treatment during allergic sensitization efficiently prevented the development of the allergic phenotype in our model. The treatment regimen used in this investigation was selected with reference to previous studies with the QY IL-4/IL-13 antagonist, where dose responses were performed and where intranasal allergic sensitization and allergic airway inflammation could efficiently be inhibited by weekly intranasal applications of 10µg of the same inhibitor together with the allergen. The inhibition of allergic sensitization with intranasal versus intraperitoneal application of identical doses of the QY IL-4/IL-13 inhibitor was equal in that study (Hahn C. et al., 2003).

The lack of therapeutic effects of an IL-4/IL-13 inhibitor as adjuvant for SIT may indicate, that the inhibition of Th2 cytokine production is not a fundamental event in successful immunotherapy but rather an epiphenomenon. This view is supported by other studies

showing that a Th2 to Th1 switch does not always occur in successful immunotherapy (Till SJ. et al., 2004)(Till S. et al., 1997)(Wachholz PA. et al., 2002)(Francis JN. et al., 2003).

An increase of IL-10 under SIT was first described by Bellinghausen et al.(Bellinghausen I. et al., 1997). In the past few years, it has been proposed that the induction of IL-10 producing regulatory T-cells is one of the key mechanisms for successful SIT since the increase of IL-10 and IL-10 producing regulatory T-cells was found to be a highly consistent finding in many studies investigating the immunomechanisms of SIT in patients (Till SJ. et al., 2004)(Akdis CA. et al., 1998). In a murine asthma model Vissers et al. demonstrated recently that subcutaneous allergen immunotherapy induced a suppressive memory responses mediated by IL-10 (Vissers JL. et al., 2004). In mice treated with monoclonal antibodies against IL-10 receptors, the beneficial effects of SIT were largely abrogated. This underlines the idea that the induction of IL-10 producing regulatory T-cells is a key mechanism for successful SIT. In line with this, our SIT model showed an increased IL-10 production in the BAL fluid as well as in spleen cell cultures. Furthermore, we found the increased number of IL-10 producing T reg cells. The SIT induced increase of IL-10 producing T reg cells, however, was absent in mice, which have been treated with the QY IL-4/IL-13 inhibitor as adjuvant during immunotherapy. Thus, IL-4 and IL-13 might be critical controlling cytokines for regulatory T-cells. Previously Akbari et al. showed that pulmonary dendritic cells, exposed to respiratory antigens transiently produced IL-10 and stimulated the development of CD4 T regulatory 1 like cells that also produced IL-10 (Akbari O. et al., 2001). The adoptive transfer of these latter cells induced an antigen specific unresponsiveness in recipient mice. Therefore, the induction of IL-10 producing pulmonary dendritic cells might be responsible for the effects of intranasal immunotherapy in our mouse model.

In conclusion we show here, that a cytokine directed immunotherapy, using an IL-4/IL-13 inhibitor as adjuvant for allergen specific immunotherapy did not enhance anti allergic effects. This supports the viewpoint that not a shift from a Th2 to a Th1 cytokine profile but other factors e.g. the induction of IL-10 producing regulatory T-cells might be key events in successful allergen immunotherapy.

5.2. The role of regulatory T cells in allergies

Over the past few years there is a increasing evidence that the regulatory T cells could contribute to suppressive and regulatory events in allergic disorders. Several different populations of T cells are described to have these regulatory properties. Recent studies described that both naturally occurring Treg cells and antigen-driven IL-10-secreting regulatory T cells have been implicated in the regulation of allergen-induced Th2 responses in mice and humans (Stassen M. eta al., 2004)(Ling EM. et al., 2004)(Akbari O. et al., 2001)(Francis JN. et al., 2003).

In human studies, the reduction of allergic symptoms after successful allergen immunotherapy was associated with the appearance of IL-10 producing T reg cells (Francis JN., 2003)(Jutel M., 2003). In animal studies, a population of CD4+ T cells induced by OVA immunization has been shown to inhibit the development of IgE responses (Curotto de Lafaille, et al., 2001). However, mouse studies on the role of Treg cells in different aspects of allergic disease and asthma are less studied (Van Oosterhout AJ. and Bloksma N., 2005).

To investigate the role of regulatory T cells in the mouse model for allergic airway inflammation, we used the superagonistic monoclonal antibodies to CD28. Recent observations argue, that superagonistic a-CD28 is able to induce an in vivo and in vitro

expansion of CD4+CD25+ regulatory T cell populations, mimicking the effects of natural ligands by costimulating the T-cell response, without TCR engagement (Hünig T. and Dennehy K., 2005).

Administration of a various doses of a-CD28 mAb in parallel with OVA sensitization, resulted in a slight increase of IL-10+ CD25+ Tcell subsets of CD4+ spleen cells. Besides, however, the mice that were treated during allergic sensitization phase showed the stronger Th2 response, than the allergic mice that were immunized only with OVA. In congruence with these phenomena are the increased OVA specific IgE titers, associated with an enhanced secretion of Th2 type cytokines and accompanied by airway eosinophilia. Moreover, the treatment of mice with a-CD28 mAb, at the sensitization phase did not show any obvious differences in the expression of FoxP3, which is a marker of naturally occurring T reg cells.

In support of a Th2-promoting role of CD28-derived signals, previously, it was noticed that the polyclonal activation of CD4 T-cells by the CD28 superagonist resulted in the expression of a functional Th2 profile, which was further supported by an increase in IL-4 producing cells in vivo (Rodriguez-Palmero M. et al., 1999). Moreover, after conventional costimulation or polyclonal activation by CD28 superagonist, but not after TCR stimulation alone, the Th2 promoting transcription factor GATA-3 was rapidly induced (Rodriguez-Palmero M. et al., 1999).

On the other hand, conflicting results were observed, when a-CD28 mAb were used at the allergen challenge phase. Here we demonstrate that administration of a-CD28 mAb in established allergic state significantly relives allergic symptoms, by reducing airway eosinophilia, decreasing OVA specific IgE titer in line with suppression of Th2 cytokines in OVA stimulated spleen cell cultures. Furthermore, therapeutic application of a-CD28 mAb significantly increases IL-10 synthesis and expands IL-10+CD25+ regulatory T cell

population as well as FoxP3+ T cell subsets. In agreement with these findings are the previous studies reporting that in vivo stimulation with superagonistic a-CD28 increased the CD4+CD25+ cell population which indeed exhibit all phenotypic and functional features of regulatory T-cells, such as high expression of CTLA-4 and transcription factor FoxP3, lack of IL-2 but massive IL-10 production (Lin C.H. and Hunig T., 2003).

Moreover, we highlight that the increase of IL-10+ T cell population in our mouse model of allergic airway inflammation was associated with the downregulation of the allergic condition and indeed underlies their anti-allergic effect. IL-10 has previously been reported to be a potential regulatory factor in allergen-induced airway inflammation. This has been shown by transfer of engineered IL-10-producing T cells which reduced AHR and inflammation in a murine model (Oh JW. et al., 2002). Furthermore, recent studies show that the transfer of allergen-specific CD4+CD25+ T regulatory cells to sensitized mice abrogates the features of allergic airway disease in vivo (Kearley J. et al., 2005).

Taking together our findings rise the idea to further study the strategies for IL-10 induction, including the expansion of the naturally occurring CD4+CD25+ population or enhancement of regulatory T cell function.

6. SUMMURY

Allergic disease are inflammatory disorders in which aberrant immune regulation occurs, and susceptible individuals mount allergen specific T helper 2 (Th2) responses, which drives disease pathology. Recent studies indicate that Th2 responses that are characteristic of allergic manifestations can be regulated by both naturally occurring CD4+CD25+ regulatory (Treg) cells and antigen-driven IL-10-secreting CD4+ regulatory T cells. Evidence is also emerging that successful Allergen specific immunotherapy (SIT) might work through the induction of IL-10-secreting regulatory T cells.

In the first part of this work, I demonstrated the efficiency of allergen specific immunotherapy in the mouse model for allergic airway inflammation. Here I could show that intranasal administration of SIT abrogates allergic symptoms more efficiently, than the subcutaneous treatment. Furthermore, an IL-4/IL-13 (QY) inhibitor was used as an adjuvant for SIT, which has been demonstrated to have an anti-allergic potential, when administered prophylactically during allergic sensitization. However, the combination therapy with SIT and the inhibitory molecule QY did not show any significant enhancement in regards to all measured allergic parameters, when compared to monotherapy with SIT. These results provide the evidence, that shift from Th2 to Th1 cytokine profile might not be a key event in successful SIT. Subsequently, the investigation of immune mechanisms under successful SIT demonstrate that the increase of IL-10 secreting CD4+ T regulatory cells is associated with the suppression of airway inflammation in our mouse system, suggesting that these T cell subsets might be involved in the regulatory mechanisms of allergic disorders.

In agreement with these findings is the second part of this work, where superagonistic a-CD28 mAb´s were used for the expansion of T regulatory cell subsets in our murine model for allergic airway inflammation. Here I could show, that the application of a-CD28 mAb

during allergic sensitization, resulted in the establishment of a Th2 state, rather than a stimulation of a Treg cell population, supporting the Th2 promoting role of a-CD28 mAb together with TCR engagement. However, interesting findings were obtained by application of the superagonistic a-CD28 mAb in the challenge phase in established allergy. Conversely to the previous experiment, therapeutic administration of a-CD28 mAb lead to the generation of IL-10 secreting CD4+CD25+ T cell population in line with the induction of anti-allergic effects.

Taking together the results of this study argue for the anti-inflammatory properties of T regulatory cells in allergic disease and highlights importance of these T cell subsets in the suppression of Th2 cell-driven response to allergen. Moreover, these observations suggest that the induction of IL-10 in vivo by T regulatory cells may represent a novel treatment strategy for allergic disorders.

7. Zusammenfassung

Allergische Erkrankungen sind Störungen, bei denen es zu Immunfehlregulationen kommt und die bei empfänglichen Individuen zur Entstehung von Allergen spezifischen T-Helfer 2 (TH2) Immunantworten führen. Neuere Untersuchungen deuten darauf hin, dass die für Soforttypallergien charakteristischen TH2 Immunantworten sowohl durch natürlich vorkommende CD4+CD25+ regulatorische T Zellen (Treg) als auch durch Antigen induzierte IL-10-secreting CD4+ regulatorische T Zellen kontrolliert werden können. Weiterhin gibt es Hinweise, dass eine erfolgreiche Allergen spezifische Immuntherapie über die Induktion von IL-10 sezernierenden T reg Zellen vermittelt wird.

In ersten Teil der Arbeit wird die Effizienz einer Allergen spezifischen Immuntherapie (SIT) in einem Mausmodel für allergische Atemwegsentzündung demonstriert. Als Allergieparameter wurden Allergen spezifisches IgE im Serum, verschiedene TH1 und TH2 Cytokine in der brochoalveolären Lavage Flüssigkeit und nach in vitro Restimulation in Milzzellen untersucht. Weiterhin wurden Histologien von Lungengewebe angefertigt, um das eosinophile Entzündungsinfiltrat und die Asthma typische Becherzellmetaplasie darzustellen. Weiterhin wurden durch FACS Untersuchungen regulatorische T Zellen nachgewiesen.

Es konnte gezeigt werden, dass im Mausmodell die intranasale Applikationsform der SIT die allergischen Symptome effizienter bekämpfen konnte, als die beim Menschen etablierte subcutane Applikationsform. Um Mechanismen zu definieren die eine SIT effizienter machen könnten wurde ein IL-4/IL13 Inhibitor (QY) als Adjuvans für die SIT benutzt. Für den Zytokininhibitor konnte gezeigt werden, dass bei einer Applikation während der allergischen Sensibilisierung die Entstehung einer TH2 Immunantwort und die Ausbildung allergischer Symptome verhindert wird. Die Applikation des Inhibitors zusammen mit einer SIT zeigte jedoch keine zusätzlichen signifikanten antiallergischen Effekte im Vergleich zur

Durchführung der SIT als Monotherapie. Diese Ergebnisse deuten möglicherweise daraufhin , dass der bekannte Wechsel einer TH2 Immunantwort zu einer TH1 Antwort während der SIT nicht der Schlüsselmechanismus zu einer erfolgreichen Behandlung ist. Insbesondere weil unter der SIT auch in unserem Mausmodell die Induktion von IL-10 sezernierenden CD4+ T regulatorischen Zellen mit der Suppression der allergischen Atemwegsentzüdnung vergesellschaftet waren, so dass möglicherwiese diese Zellen für den Therapieerfolg relevant sind .

Um die Rolle regulatorischer T Zellen im Allergiemodell näher zu beleuchten wurde im 2. Teil der Arbeit ein monoklonaler superagonistischer anti-CD28 Antikörper benutzt, von dem bekannt ist dass T regulatorische Zellen in vivo induziert werden.

Es konnte gezeigt werden, dass die Applikation des Antikörpers während der allergischen Sensibilisierung die Etablierung einer TH2 Immunantwort verstärkte. Im Gegensatz dazu wurden durch die therapeutische Applikation des anti CD28 Antikörpers in einer etablierten Allergie, IL-10 sezernierende CD4+CD25+ T Zellen induziert, welches mit einer Abschwächung der gemessenen Allergieparameter einherging.

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9. ABBREVIATIONS

APC:	Antigen presenting cells
AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage fluid
BCG:	Bacillus calmette-guerin
BMI:	Body mass index
BSA:	Bovine serum albumine
CD:	Cluster of differentiation
CTLA-4:	Cytotoxic T lymphocyte-associated protein-4
DC:	Dendritic cell
DNA:	Deoxyribonucleic acid
EARs:	Early (acute) allergic responses
ELISA:	Enzyme-linked immunosorbent assay
Eos:	Eosinophils
FACS:	Fluorescence-activated cell sorting
FCS:	Fetal calf serum
FcεRI:	Fc epsilon receptor I
FoxP3:	Forkhead box P3
H&E:	Hematoxylin and Eosin
HRP:	Horseradish Peroxidase
ICOS:	Inducible co-stimulator
Ig:	Immunoglobulin
IFN-y:	Interferon y
IL:	Interleukin
IL-4Rα:	IL-4 receptor α

i.n.:	Intranasal
i.p.:	Intraperitoneal
ISAAC:	International study of asthma and allergies in childhood
iso:	Isotype
LARs:	Late allergic responses
LPS:	Lypopolysaccharide
Lymph:	Lymphocyte
mAb:	monoclonal antibodies
macr:	macrophages
MHC:	Major histocompatibility complex
n:	Number of animals
neutr:	Neutrophils
NK:	Natural killer
NS:	Not significant
OVA:	Ovalbumin
PBS:	Phosphate buffered saline
QY:	IL-4/IL-13 inhibitor, double mutated IL-4 molecule (Q116D, Y119D)
RNA:	Ribonucleic acid
s.c.:	Subcutaneous
SEM:	Standard Error
SIT:	Allergen specific immunotherapy
STAT-6:	Signal transducer and activator of transcription 6
TCR:	T-cell receptor
Th cell:	T helper cells
TLR:	Toll-like receptor
TNF-α:	Tumor necrosis factor-α

Treg:	T regulatory cells
VCAM-1:	Vascular cell-adhession molecule 1
WHO:	World health organization

10. ACKNOWLEDGEMENTS

I would like first of all to express my deepest gratitude to Dr. Susanne Grunewald for giving me the opportunity to do my PhD study and to make an interesting research at the Dept. of Dermatology. Many thanks for supervision and her support to my work, and of course for kind advices and helps to me and my family during these years.

I am grateful to Prof. Eva B. Bröcker for all her supports, scientific discussions and kindness during being at the Dept. of Dermatology.

My deep gratitude to Prof. Hünig, first of all for giving me the opportunity to be a member of Graduate College of ``Immunomodulation``. For his useful and interesting scientific discussions. It was always a great pleasure for me to attend an interesting scientific seminars and presentations during these years. Many thanks for creating such a positive atmosphere and stimulating support.

I also would like to thank all students of graduate college for nice friendship and interesting discussions.

My gratitude to Dr. Klaus Erb and peoples from his lab. Many thanks to Gisela, Thomas, Tanya, Andrea for guiding and helping me in the beginning of my PhD work to learn all experimental procedures. Especially, I want to thank Claudia Trujillo for giving me some experimental hints, for her kind support, help and of course her lovely friendship.

My deep gratitude to Andreas Hohaus, for helping me in my hardest situations during these years. Many thanks for his nice friendship and optimistic discussions, for his kind supports and for keeping in touch.

Many thanks to Dr. Christian Hahn to giving me a useful advices in the beginning of my PhD work and for his interesting scientific discussions.

I thank Susan Schellworth-Scherz for excellent animal care, herewith I would like to thank staff of the histology and immunofluorescence laboratory for their kind attention to guiding and teaching me special experimental skills.

And my deepest gratitude to my son Sandro and my husband Rezo for being with me these years, sharing my success and fails, for there support and for keeping patience during these time.

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International Islet Training Course, Istanbul, Turkey, 1997

Immunology Training Course (Tempus-Tacis International program), Tbilisi, Georgia, 1997

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International workshop "Gene expression control in Haemato-Lymphoid cells" Wuerzburg, Germany, 2003

Presentation Technique workshop, Wuerzburg, Germany, 2004

Course of Flow Cytometry, Wuerzburg, Germany, 2004

Grundkurs Tierschutz und Versuchstierkunde, Wuerzburg, Germany, 2004

Effective Scientific Writing workshop, Wuerzburg, Germany, 2005

4th workshop "Animal models of Asthma", Hanover, Germany, 2005

Meetings/Conferences

Black Sea Diabetes Meeting, Istanbul, Turkey, 1997

34th Annual Meeting of EASD, Barcelona, Spain, 1998

2nd International conference, "Strategies for Immune therapy", Wuerzburg, Germany, 2004

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34th Annual Meeting of EASD, Barcelona, Spain, 1998
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Gogishvili T. et al., "Local intranasal Allergen Specific Immunotherapy efficiently inhibits allergic airway inflammation and induces regulatory T-cells in a murine asthma model", (Poster Presentation)

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12. LIST OF PUBLICATIONS

Gogishvili T. Wohlleben G, Erb K, Bröcker E.B, Grunewald.
Local intranasal Allergen Specific Immunotherapy efficiently inhibits allergic airway inflammation and induces regulatory T-cells in a murine asthma model. XXXII. Annual meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), 3-5 March 2005, Innsbruck, Austria. Arch Dermatol res 2005; 296:394

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