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# Role of allergen-specific CD8+ T cells in the murine asthma model

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# 1 ABSTRACT

Allergy is a disease which is present to an increasing extent in the population, especially in industrialized countries. Studies in humans and rodents have indicated a causative role for $CD8^+$ T cells in IgE-mediated allergic inflammation, but their function is still controversial. Aim of this study was to analyze the role of allergen-specific $CD8^+$ T cells during the development of allergic airway inflammation in two parallel but diverging outcome models. In murine allergy models the dose of the sensitizing antigen has previously been shown to affect the phenotype of IgE-mediated airway inflammation. Application of abundant antigen during the sensitization phase lead to a significant reduction of the inflammatory response in contrast to a low dose sensitization. Studies in both, low and high dose sensitization protocols indicated that in addition to CD4+ T cells, CD8+ T cells also have an important role in mediating and regulating allergic inflammation. In this study different markers of CD4+ T cells (e.g. CD154) and CD8+ T cells (e.g. MHC class I multimer technology) were used to analyze the induction, the natural distribution and the phenotype of allergen-specific CD4+ and CD8+ T cells in a murine C57BL/6 model of alum-ovalbumin (OVA)-induced IgE-mediated allergy. Responses in the allergy model were typically characterized by the induction of OVA-specific IgE and $IgG_1$ and airway eosinophilia following OVA allergen aerosol exposure. Conditions of antigenic overload, which resemble specific immunotherapy and antigen presentation in the context of bacterial signals, were favourable for inducing a protective and cytotoxic $OVA_{257-264}$-specific CD8+T cell response. In parallel a dramatic reduction in the number of antigen-specific CD4+ T cell and significantly reduced airway eosinophilia and total cell number in lung and BAL could be observed. Thus, the data suggests a protective role for allergen-specific effector CD8+ T cells in the regulation of airway inflammation, which may be due to their specific cytokine and migration pattern, and most importantly to the context of antigen priming. Thus, it can be concluded that allergen-specific $CD8^+$ T cells seem to protect from allergic inflammation in the lungs; their number, which is dependent on the sensitization dose, appears to be a critical predictor for the severity of the allergic phenotype.

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# 3 INTRODUCTION

## *3.1 Asthma and allergy*

There is no definition of asthma that is applicable to all cases. This difficulty reflects not only the lack of a single biological marker or clinical test for asthma but also the variable expression of symptoms, multiple aetiological factors, heterogeneous responses to treatment, and differing outcomes. Current "definitions" are in fact descriptions of the characteristics of the disease—for example, the 1995 Global Initiative in Asthma definition: *"Asthma is a chronic inflammatory disorder of the airway in which many cells play a role, in particular mast cells, eosinophils, and T lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or in the early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli."* (National Institutes of Health, Global initiative for asthma (Natl Heart Lung Blood Inst Publ no 95-3659), NHLBI, Bethesda, MD (1995)

According to the World Health Organization, more than 130 million people suffer from asthma, and the number is increasing. Allergic asthma can present for the first time at any age, but incidence is highest in childhood (Dodge and Burrows, 1980). Asthma presenting in childhood frequently remits during adolescence but can recur in adult life (Jenkins et al., 1994); asthma in adults tends to persist for life and is probably associated with an accelerated decline in lung function (Lange et al., 1998). Deaths from asthma are now uncommon, especially in children and young adults.

Allergic sensitization begins when an individual encounters and responds to a foreign protein. This may occur at a variety of sites including, the skin, circulation, respiratory, gastrointestinal, and genitourinary tracts. Sensitization is initiated by specialized antigen-presenting cells, such as dendritic cells (DC), macrophages, and B cells that take up foreign proteins, internalize them, and process them into small peptide fragments. The consequence of antigen recognition is activation, differentiation, and clonal expansion of many cells. One of those are CD4+ T helper 2 (Th2) cells, which alter in the quantity and type of surface molecules. Additionally this leads to production and secretion of many cytokines, such as IL-

4, IL-5, IL-6, IL-10, and IL-13. The result is Th2-mediated recruitment and activation of eosinophils in the skin, respiratory and gastrointestinal tracts, as well as the production of allergen-specific IgE by B lymphocytes

## 3.2 Environment and genetic background

It has been proposed that improved hygiene, which partially explains the reduced rate of infections in western countries, is at the origin of increased incidence of allergic and autoimmune diseases. This idea has originated what is called "The hygiene hypothesis, " (Bach, 2002; Strachan, 1989). The hypothesis suggests that the recent rise in allergic disease among children in affluent societies is due the preferential programming of the T cell repertoire towards pro-allergy Th2 responses, brought by the decline in infections (increased hygiene, immunization, decreased sibship size, antibiotic use) (Cookson and Moffatt, 1997). With the increasing recognition of the role of T regulatory cells (T regs) and cytokines in the pathogenesis of allergic inflammation, the hygiene paradigm has been extended recently integrating infection's role in generating such cells and mediators (Yazdanbakhsh et al., 2002).

The increased prevalence of asthma over time in the developed world seems to be part of a generalised trend of increasing prevalence of allergic sensitisation and allergic disease. Although these findings provide clear evidence that environmental factors play a major part in the cause of asthma and allergy, family studies show that a strong component of asthma risk is genetically determined. Genome screens with classical linkage and fine mapping approaches suggest that susceptibility to asthma is determined by many genes that have a moderate effect.(Wjst et al., 1999), additionally association of genes and their polymorphisms with features of asthma has been an important advance over the past decade (Bochner and Busse, 2005)

## 3.3 Th1 and Th2 paradigm

The Th1/Th2 paradigm is a cornerstone for our understanding of T cell responses (Mosmann et al., 1986). It conveniently subdivides T cell immune responses into two groups. Th1 is specialized for defence against intracellular pathogens including viruses and some intra-cellular bacteria defence and large extra-cellular pathogens such as helminths. Th1 responses

depend on IL-12 and IFN-γ to mediate a range of biological effects designed for antiviral immunity.

In contrast, Th2 responses employ the cytokines IL-5, IL-4, and IL-13, which promote the mobilization of eosinophils and cause other inflammatory processes designed to expel large parasites.

The Th1/Th2 paradigm also has particular relevance for certain inflammatory diseases, as asthma is essentially a Th2 response gone awry, and many autoimmune diseases depend on a Th1 response to autoantigens. In atopic individuals, inhalation of harmless allergens induces a preferential Th2 development, whereas in non-atopic individuals, a Th1 response predominates. The predisposition to develop a Th2 phenotype is genetically determined, whereas environmental or genetic factors can probably contribute to further consolidate the overall skewing of the Th2 response.

## *3.4 Immunity versus tolerance*

The immune system is designed to either respond or tolerate foreign antigens. A rationale justifying immune tolerance is to avoid developing immune responses to harmless inhaled and ingested proteins, while at the same time protecting the host against pathogens. Failure of the regulatory mechanisms governing immunity and tolerance, either by generating an erroneous immune response against perceived pathogens or failure to ignore innocuous proteins could potentially result in immunity directed against innocuous proteins from various sources, like pollen or house duster mites. Pathogen recognition by the immune system elicits an immune response designed to eradicate the organism. In atopic individuals, however non-pathogenic foreign proteins elicit an allergic class of response. The reason why susceptible individuals generate allergic responses to foreign antigen is unknown but appears to be dependent upon the context of the encounter.

From a therapeutic point of view, evidence has been provided that IgE-mediated, Th2 cytokine-dominated allergic diseases can be modulated towards a state of allergen tolerance either through allergen-specific immunotherapy (SIT) (Akdis et al., 2006; Gabrielsson et al., 2001) or by natural course (Blaser et al., 1998; Platts-Mills et al., 2001). Examples of natural modulation of an IgE-mediated allergic response include bee keepers who are repeatedly exposed to allergenic antigen when stung during the honey bee season (Blaser et al., 1998).

During SIT, which is clinically successful in 60-70% (e.g., in birch pollen-induced allergic rhinitis) up to 95% (e.g., in vespid venom allergy) of treated patients, immune modulation towards an IgG4- and IL-10-dominated response is accomplished by repetitive application of excessive amounts of allergenic antigen over several years (Bousquet et al., 1998; Till et al., 2004). During the maintenance phase of an SIT course, a patient receives antigen dosages that vastly exceed both, the natural exposure to the IgE eliciting antigens and the antigen amounts applied during the initiation phase of SIT (Moffitt et al., 2004). In several studies including animal models and humans, a positive correlation has been demonstrated between the antigen/allergen dose and the clinical success of SIT (Morokata et al., 2000; Ohki et al., 2005; Sakai et al., 1999; Secrist et al., 1995). In murine models the dose of the sensitizing antigen has previously shown to affect the phenotype of murine IgE-mediated airway inflammation (Morokata et al., 2000; Ohki et al., 2005; Sakai et al., 1999), e.g. application of abundant antigen during the sensitization phase constitute an interesting immunological model to analyze the cross-presentation from exogenous antigen together with the induction of allergen-specific CD8+ T cells and its relevance in the clinically context.

## 3.5 The mouse as allergic asthma model

Animal models are essential to the understanding of the genetics and pathogenesis of human diseases. The mouse is intensively used as a model system because of its similarity to humans in genome organization, development, biochemical pathways and physiology. Several mouse mutants with characteristic gene abnormalities have greatly enhanced our understanding regarding the pathophysiology of allergic reaction (Jakob et al., 2006; Sprent and Surh, 2002).

The mouse is increasingly being used for the development of in vivo asthma models, an obvious advantage is that it is a non-endangered species that offers wide availability of genetically characterised inbred strains at relatively low cost. Above all, this species allows *in vivo* application of an extremely wide diversity of immunological tools, including gene deletion technology. The use of “Knock-out” mice has obvious advantages when evaluating the functional role of a given cell or mediator in a complex situation such as cytokines, exert their effect in a paracrine or autocrine manner. However, as for other animal species, murine models also have limitations. Mice are not human and do not spontaneously develop asthma. Therefore, apart from the difficulties in transposing murine to human data, it has to be borne

in mind that mice are probably more suitable for modelling a trait associated with asthma, rather than for modelling the entire asthma phenotype. (Kips et al., 2003)

Mouse models of allergic asthma have provided important information about the condition necessary for allergen sensitization. Induction of allergic asthma by systemic immunization leading to allergen-specific Th2 immunity followed by pulmonary allergen challenge is a common basis for a number of mouse models (Epstein, 2004).
Validation of mouse models of allergic asthma requires that they resemble clinical disease. Allergic asthma is a relapsing and remitting clinical syndrome induced by allergen exposure and is characterized by episodic reversible airway obstruction. The hallmarks of disease include eosinophilia, lung inflammation, elevated serum IgE, mucus hyper secretion, and hyper reactive airways (Busse and Lemanske, 2001; Ward and Selgrade, 2007).

Asthma is a heterogeneous disease in man, and is clearly so in mice. The ongoing debate about the role of eosinophils and their contribution to airway hyper responsiveness can be witnessed in mice. There are significant differences depending on the sensitization and challenge protocol and the parameter of airway function monitored. Eosinophil localization differs from strain to strain. Many studies defining critical pathways used genetically modified animals, knockouts, or transgenics. However, when targeted by antibodies or other inhibitors, different results are often encountered. (Gelfand, 2002; Persson, 2002).

## *3.6 Modelling asthma in the laboratory mouse*

Several parameters can be measured to determine the asthmatic response in mice, among them are lung inflammation, BALF, eosinophils and immunoglobulins.
Lung inflammation
Lung inflammation is a feature of lung disease. It can be generated as a result of the immune system response against inhaling allergens. Lung inflammation in mice models, are characterized by a massive cellular infiltration in the lung parenchyma which can occur in peribronchial, perivascular or located near large airways. These regions contain predominantly eosinophils, macrophages and lymphocytes**.** One of the methods of scoring lung inflammation is quantifying the size of the infiltration areas of lung histological sections using digital images.

Bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage fluid (BALF) is a common technique developed in order to evaluate inflammatory and immune processes in the lung; it allows the quantification and differentiation of both the cellular infiltration in the airways and various inflammatory markers in the cell-free BAL fluid. In the cellular contents eosinophils, lymphocytes, macrophages and neutrophils can be differentiated through cytospin preparation, and in the aqueous phase, interleukins, concentrations can be quantified by ELISA.

Eosinophils

The primary mechanisms contributing to airway eosinophil recruitment after allergen exposure in human asthmatic subjects are still unclear but are likely to include the activation of pulmonary CD4+ T lymphocytes and their secretion of cytokines and chemokines, as well as the resulting recruitment of effector cells, including both pulmonary mast cells and eosinophils. BAL cell profiles from allergic phenotypes are characterized by an increase in eosinophils, and for many years it has been recognised that eosinophils have the potential to cause damage to the airway mucosa and associated nerves through release of granule-associated basic proteins, lipid mediators and reactive oxygen species. But eosinophils are implicated in tissue remodelling processes as well (Kay et al., 2004).

Immunoglobulins

Type I hypersensitivity involves activation of mast cells and basophils via IgE-dependent cross-linking of cell membrane Fcε receptors. Serum antigen-specific IgE and IgG1 are elevated following almost all immunization protocols. Serum antibody titters are much higher than BAL at the acute onset of allergic asthma. A number of allergic diseases, such as allergic rhino-conjunctivitis, allergic asthma or atopic eczema, are associated with increased total and specific IgE levels (Owen, 2007). But despite the obvious presence of antigen-specific IgE and IgG1 and the capacity to induce immediate-type hypersensitivity in mice, IgE and IgG1 are not always necessary for disease (Epstein, 2004; Mediaty and Neuber, 2005). For example in humans, the determination of total IgE has its limits as a single screening parameter for allergy, since a large number of patients can still develop allergic sensitization together with allergen-specific IgE antibodies in their serum without an increase in total serum IgE levels. This explains why other parameters in addition to total IgE are required in human as well as in murine allergy models.

## 3.7 CD8 T cells

At the beginning of the 90ies the perception of how the allergic phenotype is expressed in humans was based largely upon data implicating IL-4 and IFN-$\gamma$ from different CD4+ T cell subsets in the regulation of IgE synthesis in murine systems (Finkelman et al., 1990; Mosmann and Coffman, 1989). Nowadays it is well known that allergen-specific IgE synthesis is T cell dependent through cognate activation of B lymphocytes and T cell-derived cytokines (Vercelli, 2001). Growing interest in the role of the T cell in asthma arose from the concept that, in addition to participating in IgE synthesis, T-cell products might also have direct effects on the airways through the recruitment of inflammatory cells, particularly eosinophils. A number of studies showed evidence for CD4+ T-cell activation in the peripheral blood of asthmatic patients during exacerbations and either in bronchial biopsy or bronchoalveolar lavage fluid (BALF) (Jeffery et al., 1989). Considerable interest has recently been focused on the role of CD4+ T cells in asthma and allergic disorders, on the contrary in the 90ies the involvement of CD8+ T cells received less attention.

Although it is well known that exogenous antigens, including allergens, can be processed and presented in the context of MHC class I (Grant and Rock, 1992; Nelson et al., 2000), the ability of respiratory tract antigen-presenting cells to process inhaled antigen through MHC class I and induce CD8 cell inflammation has not been well defined. It is widely accepted that cellular immune responses against extracellular antigens including proteinaceous allergens are dominated by a CD4+ T cell response. CD4+ T cells are induced after appropriate stimulation by antigen-derived peptides in the context of major histocompatibility complex (MHC) class II molecules. CD8+ T cells, which provide protective immunity against various viral and bacterial pathogens by recognizing microbial peptides bound to MHC class I molecules, are considered to be less important in this context (Gavett et al., 1994; Hogan et al., 1998; Nakajima et al., 1992). Over the last years, however, several reports using either gene knock-out strategies or adoptive cell transfer in the murine model pointed out a crucial role of CD8+ T cells in allergic airway inflammation typical for allergic asthma or allergic rhinitis, as well as in IgE regulation (Holmes et al., 1997a; Miyahara et al., 2004a; Miyahara et al., 2004b; Stock et al., 2004; Thomas et al., 2001; Wells et al., 2007). Additionally , house dust mite allergen (Der p1)-specific CD8+ T cells have been identified in patients with atopic dermatitis and their presence was correlated to a favourable clinical course (Gardner et al., 2004).

The role of CD8 T cell in allergic airway inflammation is controversial, some studies focus on the fact that CD8 T cell are participating in the aggravation of the allergic airway inflammation. E.g. studies in mouse models of asthma have shown that CD8 T cells are recruited to the airways and produce IL-4, Il-5 and IL-10 upon antigenic stimulation (Stock et al., 2004). Also with CD8-deficient mice, the adoptive transfer of antigen-primed CD8 T cells are necessary for the full development of allergen-induced AHR and inflammation in a mouse model (Miyahara et al., 2004b). Others show, that when CD8 T cells are depleted before sensitization is prevented the development of AHR despite production of allergen-specific IgE and development of ICH (immediate cutaneous hypersensitivity ). Restoring CD8T cells by adoptive transfer re-establish the ability to develop AHR in those mice.(Hamelmann et al., 1996)

## *3.8 MHC multimer*

T cells through TCR recognize peptides that are bound to MHC molecules on the surface of target cells or antigen-presenting cells (APCs). When an immunodominant peptide and its corresponding MHC molecules which bind to the peptide is identified, then it is possible to design synthetic peptide-MHC complex. Monomeric peptide-MHC complex, however, binds poorly to the TCR, and multimers (typically tetramers) which make use of cooperative binding of TCRs are necessary for efficient staining (Altman et al., 1996). MHC multimer reagents conjugated with a fluorescent dye can be used in flow cytometry, allowing highly specific detection and isolation of (complex) epitope specific T cell populations directly ex vivo.

The advantages of MHC multimer assays are that they are specific and sensitive, allow surface and intracellular phenotyping, and can be combined readily with functional assays. Very small samples can be evaluated in detail (for example, organ infiltrates). Sorting and cloning of a low amount of specific T-cells has been successful. (Lim et al., 2002)
The main disadvantage of MHC multimer is that only single specificities can be analysed. This is not a problem in inbred mouse strains or human infections, such as with Epstein–Barr virus, for which immunodominant peptides are well characterized for certain HLA haplotypes , but it is a problem for infection with HIV and hepatitis C virus, in which a complex set of epitopes might be targeted by T cells.

MHC class I-peptide multimers are currently utilised to characterize $CD8^+$ T cell responses at single cell level. The generation and use of MHC class II multimer to study antigen-specific $CD4^+$ T cells appears less straightforward. Structural differences between MHC class I and class II molecules might account for the greater difficulties to standardise the generation and production of MHC class II multimer. In MHC class I molecules, the α chain alone contains the complete peptide-binding groove and it is stable in the soluble form when associated with the $\beta_2$-microglobulin chain (Garboczi et al., 1992). In contrast, both the α and β chain of the MHC class II molecules contribute to the formation of the peptide-binding site, and molecular "zippers" are necessary to guide the correct inter-chain dimerisation to stabilize the soluble recombinant αβ heterodimers. In analogy with the production of soluble MHC class I molecules, their α and β chains are separately produced in *E. coli* as denatured proteins, which are allowed to refold correctly as αβ heterodimer in the presence of a single peptide. Once refolded, however, the MHC class II heterodimer cannot exchange the peptide any longer. The latter system is generally based on insect or mammalian cells, which offer the advantage over bacteria of producing proteins in native conformation and in glycosylated form.(Moro et al., 2005). MHC multimer technology has proved to be a very powerful tool, particularly for analyzing antigen-specific CD8+ T-cells during viral infections. However, this technology has also proved useful for bacterial (Hohn et al., 2001) and parasitic pathogens (Bonelo et al., 2000). However, this immuno-technology has not yet been fully introduced in the field of allergy (Macaubas et al., 2006; Seneviratne et al., 2002)

Previous studies have tried to identify the role of allergen-specific CD8+ T cells by either adoptive transfer or depletion experiments (Holmes et al., 1997a; Miyahara et al., 2004a; Schaller et al., 2005; Stock et al., 2004). However, neither technique allows determining the role of these cells in the natural course of IgE-mediated allergy. We therefore used H2-Kb SIINFEKL-multimers to follow allergen-specific CD8+ T cells in both, the 10µg-OVA dose and the 10mg-OVA dose long-term-model.

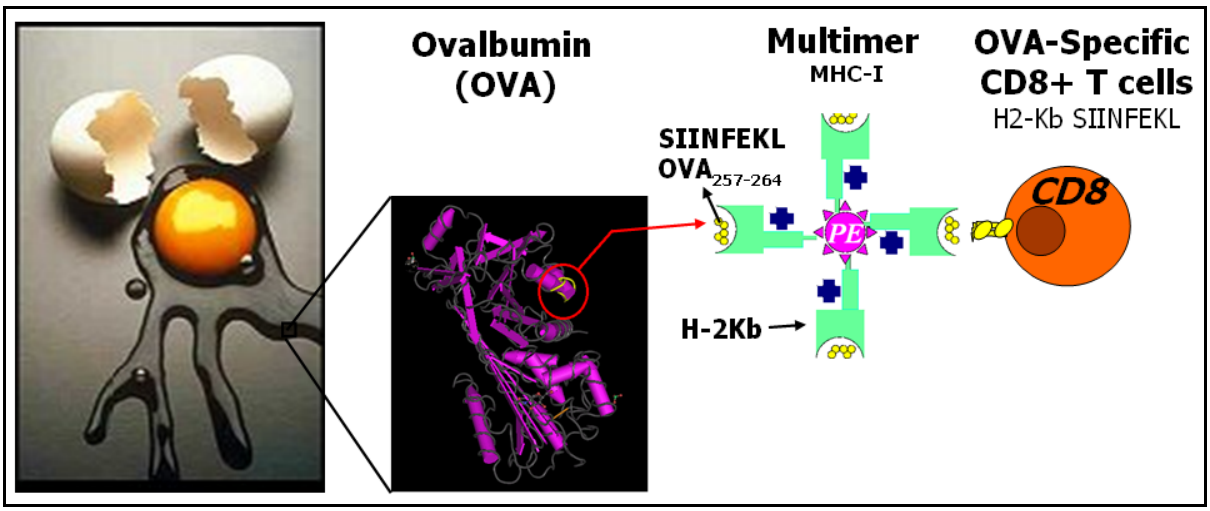


**Fig. 1 Multimer for SIINFEKL**

Schematic representation of a MHC multimer. A multimer for SIINFEKL is a structure of the MHC class I complex with a loaded peptide. In this illustration, the MHC class I multimer is bond to the SIINFEKL peptide, which is the immunodominant peptide for Ovalbumin in C57BL/6 (H-2Kb haplotype mice). This multimer (typically tetramers) will bind to the CD8 T cell which recognises this specific peptide.

# 4 AIM OF THE STUDY

This project is part of a broader project currently in progress for developing new techniques and novel therapies to control allergy. Its objective is aimed at giving new knowledge on the behaviour of CD8 T cells in atopy in general with special emphasis on the murine asthma model.

To understand the development of CD8+ T cells in allergic diseases, the functional effect or/and the particular migratory distribution of these cells will be investigated with the help of H2-Kb SIINFEKL multimers, which contain the OVA-derived peptide SIINFEKL. A detailed analysis of these specific lymphocytes in different settings e.g. at different times of the systemic immunisation, detection in different tissues (spleen, lymph nodes, peripheral blood, bone marrow, lung), using state-of-the-art methods for adoptive transfer of cells, and the quantification of other parameters involved in inflammatory process, like cytokines, cells classification and immunoglobulin levels measurement will give a panoramic overview of how these cells play a role along the clinical manifestation of the allergic response.

This new perspective on the CD8+ T cells in allergy tries to explore the contribution of these particular lymphocytes in the allergic response which, so far, has been overshadowed by the well known role of CD4+ T cells. The purpose of this thesis is to clarify the inconsistent and contradictory data on CD8+ T cells, previously generated by other researchers in the field of allergy.

# 5 MATERIALS AND METHODS

## *5.1 Animals*

The **C57BL/6** mouse was originally derived by Little in 1921. Today, it is one of the most widely used and most popular strains in both animal research and the development of mutant mouse lines. Female mice have shown in the past to have more susceptibility to develop allergic airway inflammation and a higher level of IgE in serum than male mice after sensitization and challenge, that is the reason why female wild-type C57BL/6 mice are ideal. Additionally, female mice are less aggressive than males, which helps the housing in a laboratory (Melgert et al., 2005; Van Loo et al., 2003). Those mice were initially obtained from Charles River Laboratories. Because C57BL/6 have H2Kb HLA class I haplotype, the T cells are able to recognize the immunodominant ovalbumin peptide SIINFEKL ($OVA_{257-264}$). The precursor frequency of naive CD8 T cells specific for a peptide has been estimated 1 in 2 x $10^5$, so there are about 100 to 200 T cells that recognize the immunodominant ovalbumin peptide SIINFEKL ($OVA_{257-264}$) in the entire mouse body. (Blattman et al., 2002)

The **OT-1** (C57BL/6 background) mice contain transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes. The transgenic T cell receptor was designed to recognize the immunodominant ovalbumin peptide SIINFEKL ($OVA_{257-264}$), in the context of H2Kb. Most of the CD8+ T cells from OT-1 (95%) are recognized by the MHC H2-$K^b$ SIINFEKL multimers and like most TCR transgenics these mice are somewhat immunodeficient. Those mice were initially obtained from Charles River Laboratories.

**TAP1-/-** ($Tap1^{tm1Arp}$, B6.129S2-Tap1tm1Arp/J) mice are defective in the stable assembly and intracellular transport of class I molecules. They show severely reduced levels of MHC class I surface molecules. TAP1-/- have normal numbers of CD4+ and CD4+ CD8+ T cells but are deficient in mature CD8+ T cells. This phenotype is similar to human beings with TAP1 and TAP2 deficiencies. Despite the small pool of peripheral CD8+ T cells in TAP-/-, studies have shown that these CD8+ T cells have preserved effector activity (Marrero et al., 2005). This strain should be housed under pathogen free conditions similar to the OT-1 mouse or any other immunodeficient strain. Those mice were initially obtained from the Jackson Laboratory.

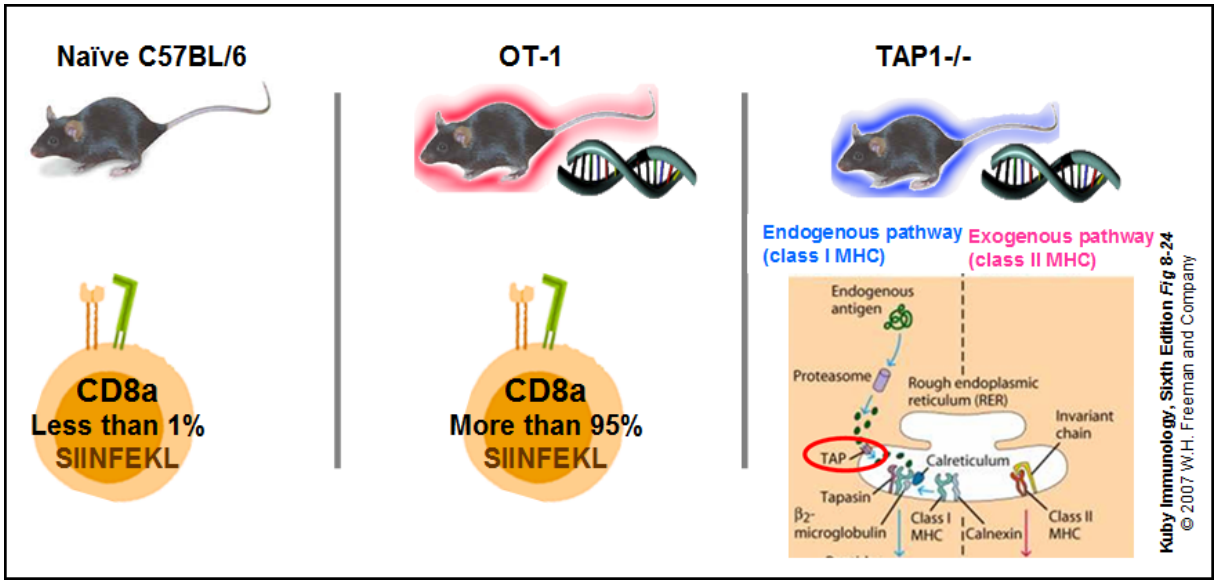


**Fig. 2 Transgenic mice**

C57BL/6 WT mice, together with two transgenic mice are used in the following work: the OT-1 mice have the ability to recognize the SIINFEKL peptide in almost all lymphocytes, the TAP1-/- are immunodeficient mice with defective intracellular transport of class I molecules. All mice were housed in HelmholtzZentrum München (German Research Center for Environmental Health; Neuherberg, Germany) pathogen-free animal facility. The study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the local government (Regierung von Oberbayern) and the Animal Care and Use Committee of the HelmholtzZentrum München Research Center.

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## *5.2 Sensitization and airway challenge*

Based on previous experiences with different protocols tested the following protocols that combine sensitization phase and aerosol challenge generating a clear outbreak of the disease were chosen. These protocols generate a notorious amount of CD8 T cells which were subject of the studies.

Mice (6-10 wk) were sensitized by intraperitoneal injection of 10 µg or 10 mg of OVA (Sigma) emulsified in 2 mg of alum (Pierce) on days 1, 10 and 26 in 200 µl PBS. Mice were subsequently challenged over six days with two rest-days in between by inhalation exposure to aerosols of OVA (6% OVA in saline for 1h using ultrasonic nebulization) (Fig. 3 and Fig.

4). One day after the last OVA-aerosol challenge the animals were sacrificed, followed by the collection of organs for further analyses. Control animals received a sensitization with OVA-i.p. but instead of OVA-aerosol challenge those animals were exposed to PBS-aerosol. A second group of control animals received equal volumes of PBS at the same points of sensitization but were exposed to aerosol of OVA (6% OVA in saline for 1h using ultrasonic nebulization). The initial data refers to a sensitization using 10µg-OVA or 10mg-OVA, in a short (2-6 weeks) or long (9-20 weeks) sensitization protocol, later on the long protocol was chosen due to the advantage of the reliability of its results, and finally the high dose 10mg-OVA was used because of its relevant role in the generation of allergen-specific $CD8^+$ T cells.

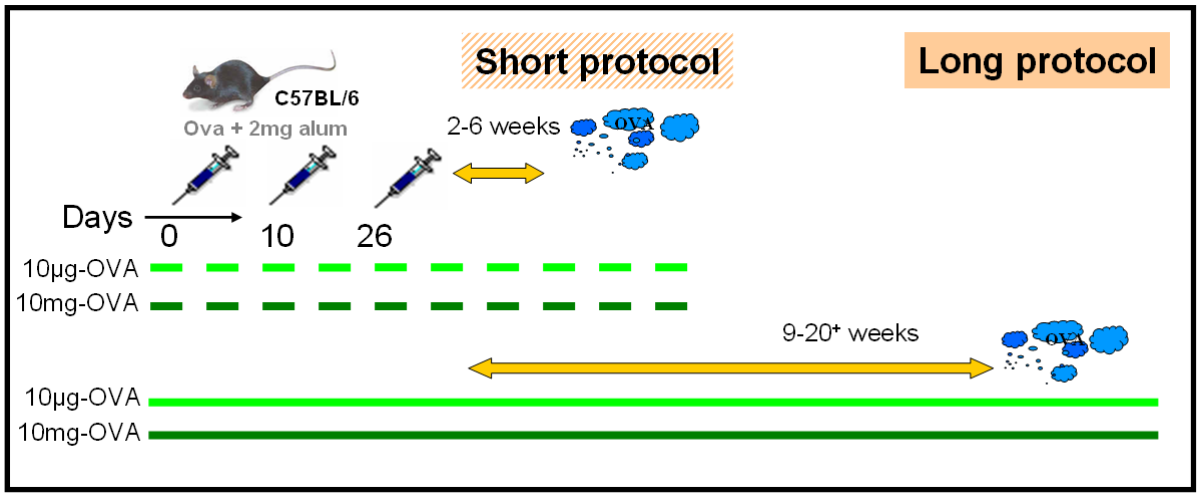


**Fig. 3 Sensitization protocols**

Here is a scheme of the different protocols of sensitization that have been used. In brief three i.p. injections of OVA-alum (10µg or 10 mg on day 1, 10 and 26 in 200 µl PBS) and later a OVA aerosol challenge, shortly after sensitization (2-6 weeks) or with a long period in between (9-$20^+$ weeks).

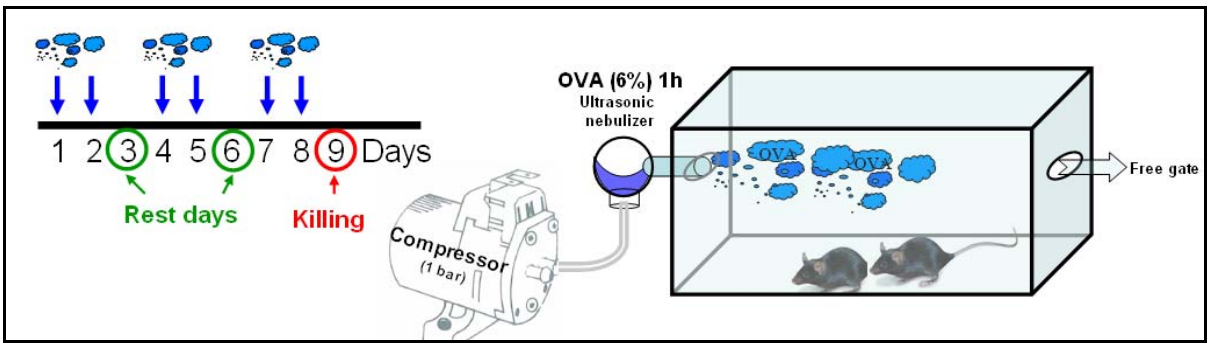


**Fig. 4 Aerosol challenge**

Illustration of aerosol challenge. Mice were subsequently challenged over six days with two rest-days in between by inhalation exposure to aerosols of OVA (6% OVA in saline). The animals were grouped and placed in a small acrylic box for exposure during 1h and using ultrasonic nebulisation (1 bar).

### 5.3 Cell preparation

To study bronchoalveolar lavage (BAL) samples a slightly modifiied protocol from Allessandrini's works was used (Alessandrini et al., 2006; Jakob T, 2006). In brief, after euthanization, the animal's chest was opened and lungs were rinsed with 800 µl of sterile PBS instilled through the trachea, cannulated into the lungs and recovered. Total cell counts were determined from BAL-fluid using a hemocytometer. After centrifugation, BAL supernatant was collected and stored at -70°C for further analysis and cell pellets were resuspended in PBS. Parts of the sample were separated for flow cytometric analysis. Cytospins were prepared for each sample by centrifugation of 150 µL BAL fluid at 400 rpm for 10 minutes. After fixation, cytospins were stained with Diff-Quik (Dade). The cells were classified as neutrophils, eosinophils, lymphocytes or macrophages using standard morphological criteria.

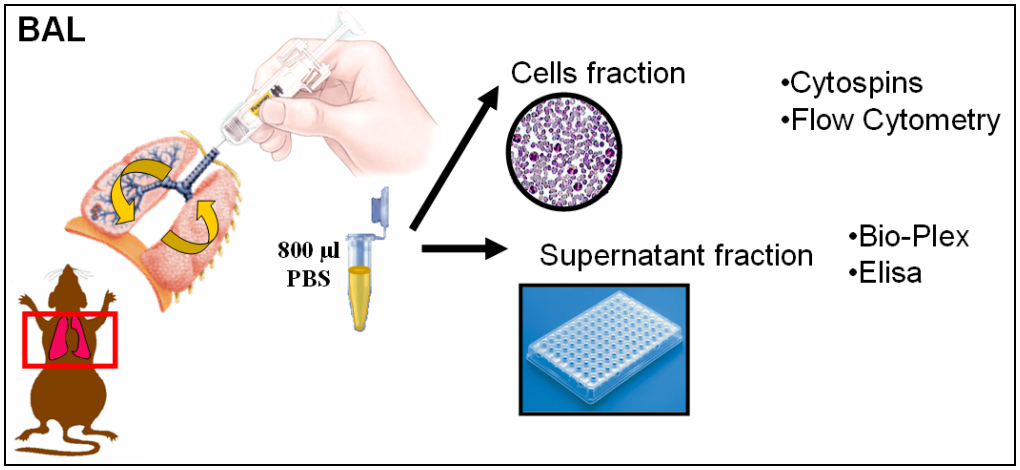


**Fig. 5 Bronchoalveolar lavage**

BAL fluid was obtained by rinsing 800 µl of PBS through the trachea into the lungs of sacrificed animals and recovering it. Cell fraction was quantified and determined using a hemocytometer and Flow cytometry analysis. The cytokine and protein content of the supernatant fraction was analyzed with Bio-Plex and/or Elisa methods.

For immunophenotyping of CD8+ T cells, lungs and livers were perfused with PBS supplemented with 10 U/ml heparin. Small pieces of tissue were incubated in 5 ml of HBSS (Gibco) containing 0.5 M EDTA for 30 min at room temperature, then washed with PBS and incubated in 150 U/ml collagenase VIII (Sigma) in a final volume of 5 ml RPMI 1640 (Gibco) containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5% (V/V) FCS at 37°C for 30 min. Subsequently, digested lung- or liver- tissues were mechanically dispersed through a wire mesh and were resuspended in PBS containing 10 % FCS. Spleens and isolated lymph nodes (cervical, para-tracheal, mediastinal, axillary, inguinal, and mesenteric) were removed and harvested by dissociation through a wire mesh. Bone marrow cells were obtained by flushing

tibial and femoral bones with RPMI 1640. To the peripheral blood and bone marrow, erythrocytes were lysed with ammonium chloride and were subsequently resuspended in PBS containing 10 % (V/V) FCS.

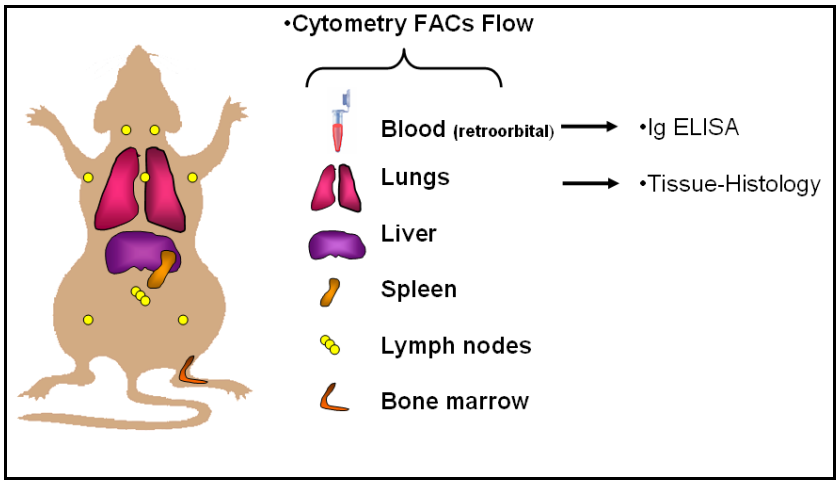


**Fig. 6 Tissue isolated from Organs**

Lungs and livers were collected from sacrificed animals and cell preparations were performed using collagenase VIII for the digestion of the tissue. Cells in other organs were isolated by mechanical dispersion through a wire mesh. In most of the experiments cell preparations were directly analyzed, in some other, cell suspension was cultivated and/or stimulated for a short period of time.

## *5.4 Determination of the degree of histological inflammation*

Lungs were fixed in 4% formalin, embedded in paraffin and cut for light microscopic examination, 3-5 µm sections were stained with H&E, Giemsa, and periodic acid Schiff (PAS), and analyzed. From each mouse, three different histology sections from lung with a total size of 1.550.000.00 µm² were digitally photographed (10x magnification). The dense peribronchiolar mononuclear cell infiltrates were visualized in darker colour, and quantified (in µm²) using the *Leica Image Manager IM-1000* software; the average of cell infiltrated area of each animal is estimated from the three pictures. And the mean of the mice group is compared.

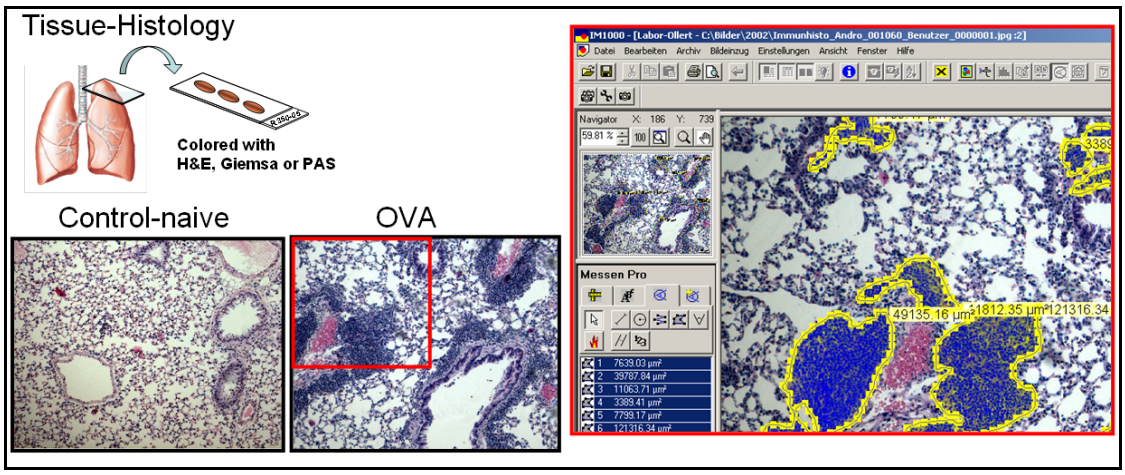


**Fig. 7 Determination of the degree of histological inflammation**

H&E-stained lung sections were scanned with a digital camera and the areas of inflammation measured using the *Leica Image Manager IM-1000* software. A representative area of the lung was chosen for measurement, and three segments of 1.550.000.00 µm2 of lung tissue were analyzed per animal. The dense peribronchiolar mononuclear cell infiltrates are visualized in darker colour, and the yellow line corresponds to the area selected as positive for cell infiltrates.

## 5.5 Determination of interleukins and immunoglobulin titers

IL-2, IL-4, IL-5, IL-10, IL-12, IL13, IFNγ and TNFα Interleukin concentrations in BAL fluid were measured by a cytokine kit from the Luminex multi-analyte system (Bio-Plex; Bio-Rad) Kit (Mouse Th1/Th2 Panel (171-F11081), that is a bead-based assay, that has the ability to measure many interleukins at once with a small amount of sample. Plasma was analyzed for total IgE, OVA-specific IgE and IgG1 using an isotype-specific sandwich ELISA (Alessandrini et al., 2006). In brief, microtiter plates (96-well) were coated with 10 µg/ml anti-mouse-IgE rat monoclonal IgG (The Binding Site) to detect total IgE, or coated with 1 mg/ml OVA to detect OVA specific IgE and IgG1 in carbonate-bicarbonate buffer (Sigma). Serum samples were diluted 1:10 for total IgE and OVA-specific IgE and 1:100,000 for OVA-specific IgG1; standards for murine IgE (clone-C382, Pharmingen) or for murine IgG1 (clone-OVA-14, Sigma) were appropriately diluted. As secondary antibodies, biotinylated rat anti-mouse IgE (clone R35-118, Pharmingen) and rat anti-mouse IgG1 (clone A85-1, Pharmingen) were used followed by incubation with streptavidin-peroxidase (Calbiochem) and 3,3'5,5'-tetramethylbenzidine (Fluka). Plates were analyzed using a standard micro well ELISA reader at 450 nm. The protein concentration was determined by using a Coomassie protein assay reagent kit (Pierce), which is a, colorimetric method for total protein quantification. Additional Immunoglobulin from plasma samples were measured using Beadlyte anti-mouse multi-immunoglobulin isotyping kit (Upstate-Millipore)

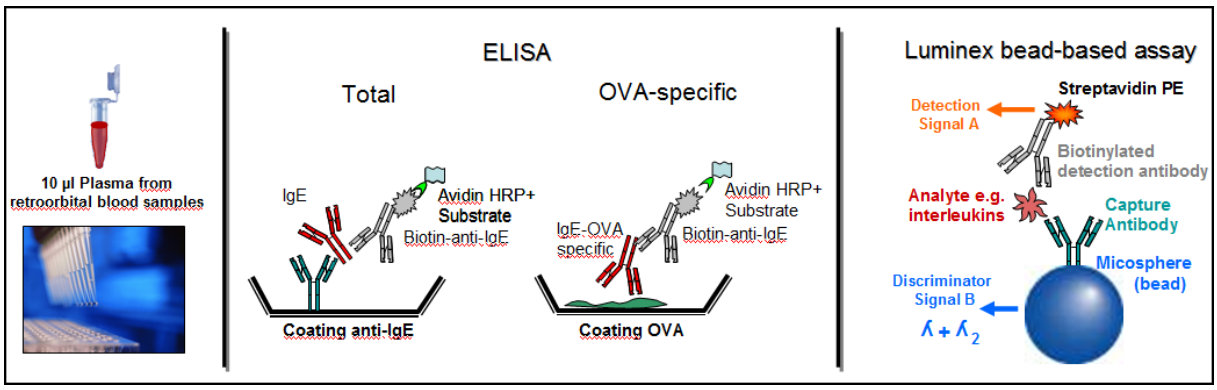


**Fig. 8 Determination of interleukins and immunoglobulin titers**

Plasma was analyzed for total IgE, OVA-specific IgE and OVA-specific IgG1 using an isotype-specific sandwich ELISA. BAL fluid was measured with a cytokine Luminex multianalyte system (Bio-Rad), additional immunoglobulins were measured with multi-immunoglobulin isotyping kit (Upstate-Millipore)

## *5.6 Adoptive cell transfer and CFSE labeling*

CD8+ T cells were positively selected from spleens of OT-I donor mice using anti-CD8 conjugated magnetic beads (Miltenyi Biotec). The selected fraction contained >90% CD8+ T cells as determined by FACS analysis. CD8 T-cell-enriched fractions were labeled with CFSE (0.5 µM final concentration in PBS) (Molecular Probes) by incubation for 10 min at 37°C. After labeling, FCS was added to a final concentration of 10% and cells were subsequently washed with PBS at 4°C. Next, cells were resuspended in 250 µl of PBS and 3 x $10^6$ CD8+ T cells were injected into the tail vein of recipient wild-type C57BL/6 mice or TAP1-/- mice 24 h before OVA-aerosol challenge.

**In vivo cytotoxicity assays** with SIINFEKL-loaded and CFSE-stained splenocytes from naive C57BL/6 mice were essentially performed as described earlier (Hamm et al., 2007; Heit et al., 2005). Equal numbers of differentially labeled cells were injected *i.v.* into OVA-sensitized animals (Fig. 9 ). After 15 h and 48 h, retro orbital blood samples were collected and CFSE-positive cells were monitored by flow cytometric analysis.

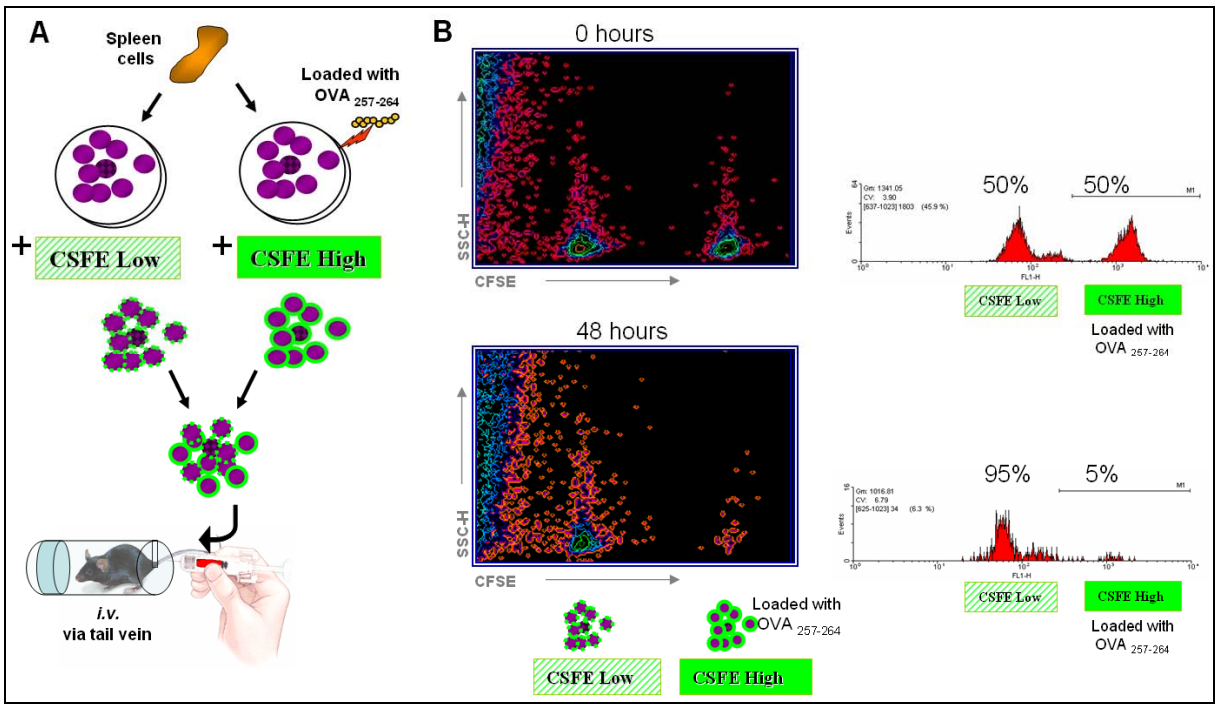


**Fig. 9 In vivo cytotoxicity assays with SIINFEKL-loaded and CFSE-stained**

A) Cytotoxic potential was demonstrated by in vivo lysis of highly CFSE-labeled and SIINFEKL-loaded splenocytes in comparison to low CFSE-labeled and unloaded SIINFEKL when adoptively transferred to a sensitized mouse. B) Cytometric analysis from CFSE-positive cells from blood were monitored after 15 h and 48 h.

## 5.7 Cytokine analysis from in vitro cell culture

Total spleen or lung cell suspensions from sensitized mice or naïve mice were cultured at a density of $2\times10^6$ cells/well in 200 µl RPMI 1640 containing 10% FSC at 37°C in 5% $CO_2$ in the presence or absence of $OVA_{257-264}$ peptide (SIINFEKL) (1 µg/ml) for 6 h. Thereafter, GolgiPlug (BD Bioscience) was added at a final concentration of 1 µl/mL in the cell culture medium during the last 5 h of the culture period. After culture, cells were washed in FACS buffer, and stained for intracellular IL-4, IFN-g, IL-10, and IL-5 using cytokine specific monoclonal antibodies (all from BD Bioscience) together with the Cytofix/Cytoperm Plus kit (BD Bioscience) according to the manufacturer's instructions.

## 5.8 MHC class I multimer staining and phenotyping of CD8 T-cells by FACS analysis

H2-Kb SIINFEKL multimer reagents were generated as described (Busch et al., 1998) or purchased from Immunotech. Cells were resuspended in FACS buffer followed by blocking of Fc receptors with excess of Fc-block reagent. To screen for cell death, cells were incubated

with ethidium monazide (Molecular Probes) or 7AAD (BD Pharmingen) followed by H2-K$^b$ SIINFEKL multimer and surface marker staining for 45 min at 4°C. The following mAbs were used: anti-CD8a (Caltag), anti-CD127 (eBioscience), anti-CD62L, anti-CD25, anti-CD69, anti-CD44, anti-Ly6C and anti-CD154 (all from BD Bioscience). Data were acquired on a FACS-Calibur (Becton Dickinson) and were further analyzed with CELLQUEST V.4.02 or FlowJo V. 7.2.2 software. Ultimately, experiments involving absolute counting of T cells (TruCOUNT Tubes; BD Pharmingen) and intracellular activation markers such as anti-CD154 were performed in a LSRII (Becton Dickinson) and were further analyzed with FACS-DiVa software and Flowjo (V.7.2.2)

## 5.9 *In vivo CD8+ T cell depletion*

CD8+ T cells were depleted *in vivo* by *i.p.* injection of purified 0,1 mg/mouse rat anti mouse IgG mAb (prepared from 2,43 hybridoma TIB-210$^{TM}$ ATCC®) two days before and on one day during OVA aerosol Challenge (see Fig. 10). The efficiency of the depletion was monitored by measurement of the remaining undepleted cells using flow cytometric analyses from orbital blood samples and CD3+ and CD8+ T mAbs staining.

In addition, two *i.p.* injections of this antibody with 100µg or 200µg per mouse were administered to prove the efficiency of the depletion *in vivo*. The CD8+ T cell depletion experiment in mice was monitored during 16 days with the help of flow cytometric analysis, where blood from WT mice was stained with CD3 and CD8 antibodies. (Fig. 30).

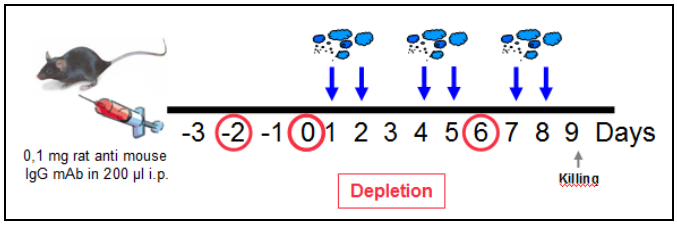


**Fig. 10 In vivo CD8+ T cell depletion**

CD8+ T cell were depleted from a group of sensitized mice by injection of purified (0,1 mg/mouse) rat anti mouse IgG mAb (prepared from 2,43 hybridoma TIB-210$^{TM}$ ATCC®) on days -2, 0, before aerosol challenge and day +6 during aerosol challenge, depletion was monitored by CD3+ CD8+ flow cytometric analyses.

## 5.10 Statistical analysis

Comparisons of groups were performed with a t test and *p* values, two tailed distribution and homoscedastic for significance were set to * = P< 0.05. ** = P< 0.01. *** = P< 0,001; data was evaluated by individual experiments, or with the collection of independent experiments. The data base was analysed using SigmaPlot for Windows v.11.0 Systat© Software, Inc. or Microsoft ® Office Excel 2003.

# 6 RESULTS

## 6.1 Characterization of low and high dose models of OVA sensitization

There is no unique standard protocol defined for the allergic airway inflammation model in mice. Different protocols vary, depending on the aim of the research. To conduct this study, many experiments were performed testing different doses of antigen in the sensitization phase and the varying periods of time between sensitization and challenge in order to design an optimal protocol.

The focus was, to design a system capable of generating a clear-cut degree of the illness and the possibility to modulate its outcome, which is displayed in changes of cells or cytokine subtypes and tissue infiltration, among others (e.g. eosinophile percentages, area of infiltration, absolute counting of cells recruitment).

### 6.1.1 Dose of OVA for sensitization

As it was already disclosed in the introduction, the dose of the sensitization antigens affect the phenotype of airway inflammation. (Morokata et al., 2000; Ohki et al., 2005; Sakai et al., 1999). This hypothesis was confirmed when mice were sensitized with 10µg, 0.5 mg, 2 mg and 10 mg of OVA and exposed to three challenges of OVA-aerosol. The allergic inflammation was present in all sensitized and challenged mice, but different doses of antigen reveal differences in the severity of the disease, which can be seen along the cells recruitment and the eosinophilia present in the BAL after aerosol challenge (Fig. 11).

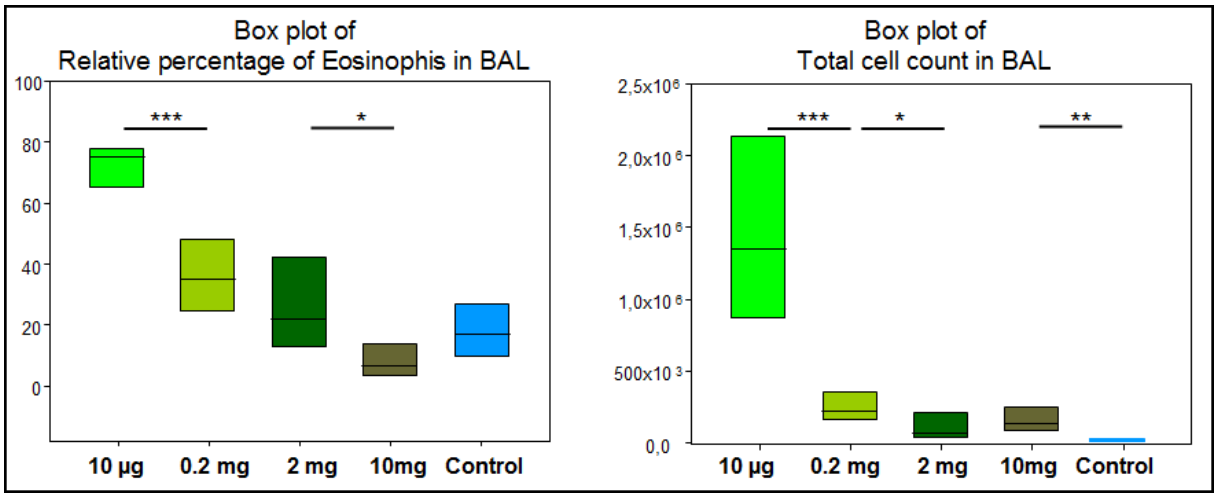


**Fig. 11 Antigen dose-dependend allergy phenotype**

The modulation of the severity of the disease was feasible using different doses of antigen (OVA) in the sensitization phase. Changes of the relative percentage of eosinophils and total cell count in BAL

were dosed-depended related. Lower doses (10µg) caused a stronger inflammation contrary to higher doses (10mg) where the severity of the disease was discreet.
Cell count in BAL of mice *i.p*. sensitized on day 1 and 7 with OVA-Alum (groups: 10µg, 0.2 mg 2 mg and 10 mg) and exposed to an aerosol challenge of 1% for 15 min. on days 68-71-74. Control animals were not sensitized but challenged with OVA aerosol. n= 5 to 7 animals per group. * = P< 0,05. ** = P< 0,01. *** = P< 0,001

The fact that low dose OVA sensitization (10µg) generated a stronger inflammation in the allergy model contrary to higher doses (10mg) which caused a minor inflammation, gave an ideal model to study the role of the immune system when the severity of the disease is modulated using different antigen concentrations. To comprehend the cause of this different outcome it is crucial to analyze the different cell subtypes and other immunologic parameters that can play a role and put up with different responses against same antigen.

### 6.1.2 Time between sensitization phase and aerosol challenge

Preliminary experiments were performed to disclose whether time between the phase of sensitization and the phase of challenge is an issue. The results obtained from short (2-6 weeks) or long (9-20 weeks) time between sensitization and challenge phase did not show major differences. Total IgE and OVA-specific IgE were significantly higher in low dose OVA (10 µg) sensitized mice than in high dose OVA (10 mg) and correlated in the same way when short protocol (short p.) or long protocol (long p.) were performed (Fig. 12 and Fig. 13). IgE, eosinophilia and the amount of infiltrated cells found in the BAL (Fig. 14) reveal that the time between the sensitization phase and the aerosol challenge is not relevant for the allergic response.
Although no major differences were found between short and long protocol, the advantage of using long period of time between sensitization and challenge can be the fully maturation of the memory cells. That is why the following experiments are obtained from challenges using a long (9-20 weeks) protocol.

To obtain an adequate recruitment of cells in BAL and a clear response of antibodies, 6 days of aerosol challenge at 6% OVA V/V 1h were optimal. Nevertheless this challenge is intense, that is why two days rest in between was given (Fig. 14). After all, 8 days from the first aerosol challenge to the sacrifice of the animal were enough to detect significant changes of the levels of immunoglobulins in blood, to recruit cells and increase the amount of cytokines in BAL.

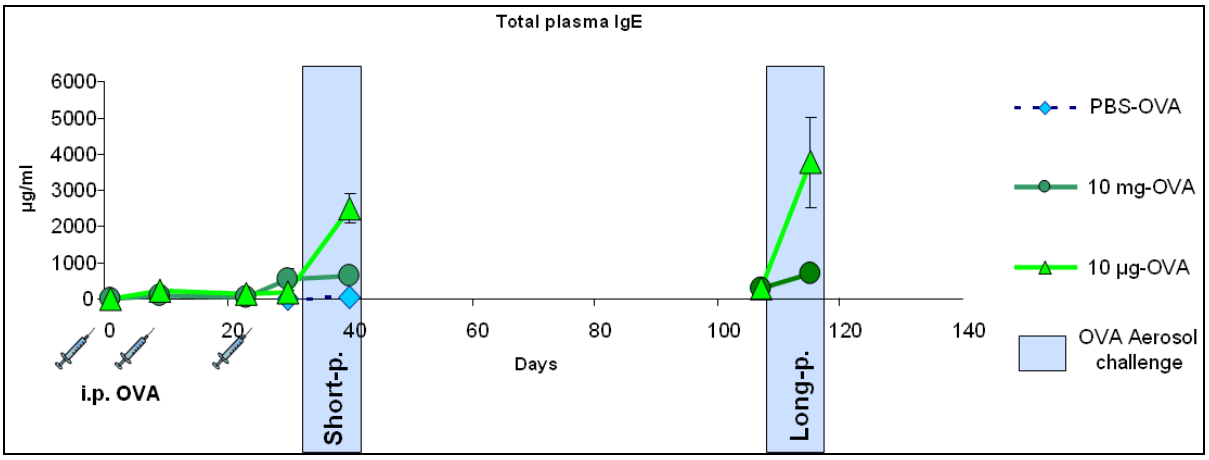


**Fig. 12 Measuring of total IgE levels in plasma samples**

Low dose OVA (10µg) generated higher levels of IgE in comparison to high dose OVA (10 mg). This phenomenon was observed short after as well as long after the sensitization phase. Two groups of sensitized mice, one with low doses of OVA (10µg) and another with high doses of OVA (10mg) were sensitized three times on days 1, 10, and 26. Mice of each group were split to be challenged either short after the sensitization phase (short protocol) or long after (long protocol). The challenge consists of 6 days OVA-aerosol, 6%, 1h with two days rest in between. Blood samples were taken during the sensitization phase and immediately before and after the aerosol challenge and values of IgE were measured by ELISA.

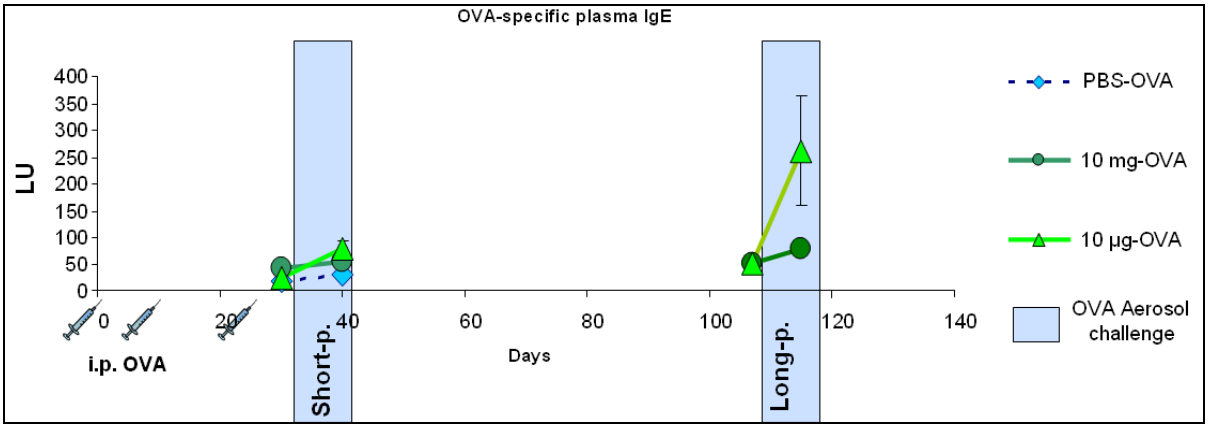


**Fig. 13 Measuring of OVA-specific IgE levels in plasma samples**

Low dose OVA (10µg) generated higher levels of OVA-specific IgE in comparison to high dose OVA (10 mg). This phenomenon was observed short after as well as long after the sensitization phase. The difference between high or low dose was remarkably higher in the long protocol. Two groups of sensitized mice, one with low doses of OVA (10µg) and another with high doses of OVA (10mg) were sensitized three times on days 1, 10, and 26. Mice of each group were split to be challenged either short after the sensitization phase (short protocol) or long after (long protocol). The challenge consists of 6 days OVA-aerosol, 6%, 1h with two days rest in between. Blood samples were taken during the sensitization phase and immediately before and after the aerosol challenge and values of OVA-IgE were measured by ELISA using lab units OVA-IgE standards (LU).

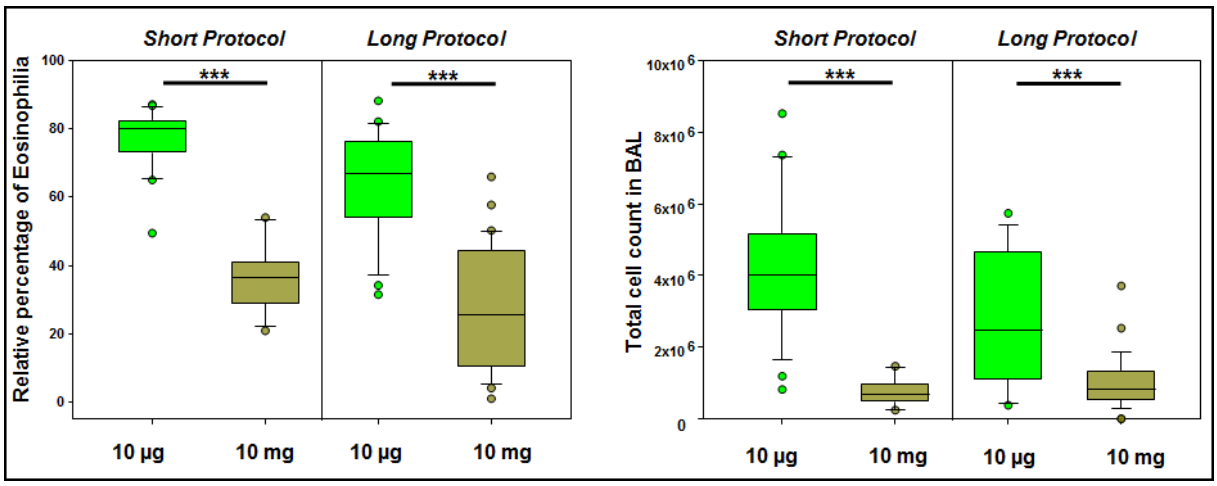


**Fig. 14 Eosinophilia and total cell count in BAL**

*Right* : **Relative percentage of Eosinophilia**. In low dose OVA (10µg) sensitization, Eosinophilia in BAL was about 70-80%, while in high dose OVA (10 mg) sensitization values were significantly lower (30-20%). This phenomenon was observed short after as well as long after the sensitization phase. *Left*: **Total cell count in BAL.** In Low dose OVA (10µg) sensitization, total cell count in BAL was about 4 x $10^6$ cells per mouse while in high dose OVA (10 mg) sensitization values were below 1 x $10^6$ cells per mouse. This phenomenon was observed short after as well as long after the sensitization phase. Two groups of sensitized mice, one with low doses of OVA (10µg) and another with high doses of OVA (10mg) were sensitized three times on days 1, 10, and 26. Mice of each group were split to be challenged either short after the sensitization phase (short protocol) or long after (long protocol). The challenge consists of 6 days OVA-aerosol, 6%, 1h with two days rest in between. BAL samples were taken 24h after the last aerosol challenge; cells were classified using standard morphological criteria by cytospins. Collection of data from independent experiments, n= 10 to 35 per group. * =P< 0,05. ** =P< 0,01. *** =P< 0,001.

### 6.1.3 Comparison between low and high dose sensitization

Both, low OVA (10µg) and high OVA (10mg) i.p. injections and subsequent challenge by inhalation exposure to OVA-aerosols (Fig. 3 and Fig. 4) led to an increase in total OVA, OVA-specific IgE (Fig. 12 and Fig. 13) eosinophilia and number of lung infiltrating cells in BAL (Fig. 14).

The localization of infiltrated cells in the lung tissue (Fig. 15) confirm the occurrence of inflammatory allergy and together with a further detailed analysis in BAL by cytospins using morphological criteria (Fig. 16) provide the different subtypes of cells raised after challenge.

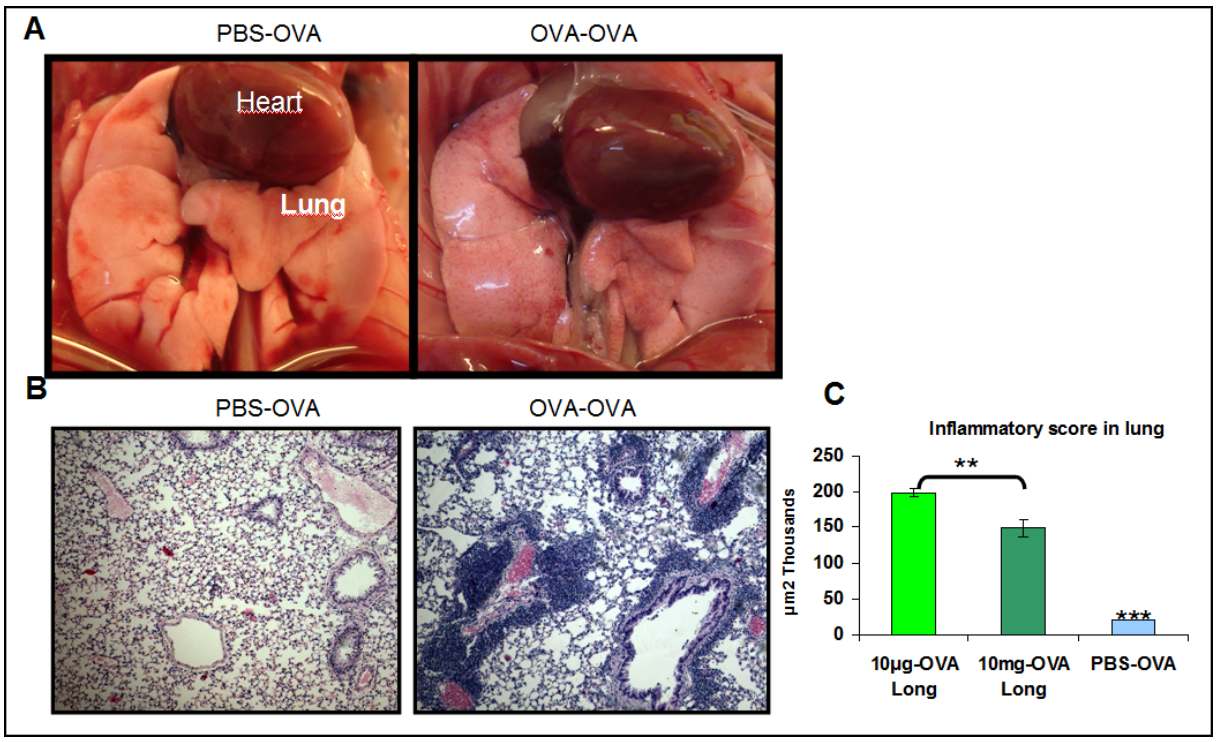


**Fig. 15 Inflammatory score in lung**

A) Mice not sensitized but challenged with OVA (PBS-OVA) did not show a visually apparent inflammation in the histology slide, contrary, to the mice sensitized with OVA (either 10 µg or 10 mg) and challenged with OVA-aerosol (OVA-OVA), were lungs are clear darker, and presenting dotted hemorrhages. B) Lungs were prepared for histological analysis using H&E-staining, then scanned with a digital camera and a representative area of the lung was chosen for the measurement of infiltrated cell areas. The dense peribronchiolar mononuclear infiltrated cells are visualized in darker colour. C) Significant differences between Low (10µg-OVA) and High (10mg-OVA) can be observed when the inflammatory scores are compared. PBS-OVA control animals show a minimum inflammatory score. Mice were sensitized on days 1, 10, and 26 with low dose OVA (10µg) or with high dose OVA (10mg). The challenge conditions consist of 6 days OVA-aerosol 6% 1h with two days rest in between. Lungs were dissected 24h after last aerosol challenge. * =P< 0,05. ** =P< 0,01. *** =P< 0,001

Cytokines from BAL supernatant were measured in both protocols using bead-based assay (see section 5.5 p.22 ), here, the contents of IL-4 found in BAL fluid are correlated with the amount of eosinophils in BAL which generate a positive correlation and it is easy to distinguish each of the two groups of low and high dose sensitization (Fig. 16 page 32). Finally, all this data corroborate that the severity of the disease is remarkably minor in high dose than in low dose sensitized animals; and those results agree with the findings described by others (Morokata et al., 2000; Ohki et al., 2005; Sakai et al., 1999).

Cytokine levels measured in BAL fluid from sensitized mice of which the CD8+ T cells were depleted reveal a significant increase of IL-4, IL-10 and IL-12 levels (Fig. 31). Meta-analysis

of eosinophils & IL-4 in BAL comparing the two protocols high dose and low dose, and depleted CD8+ T cells, gives clear evidence of clustering of the different protocols (Fig. 32).

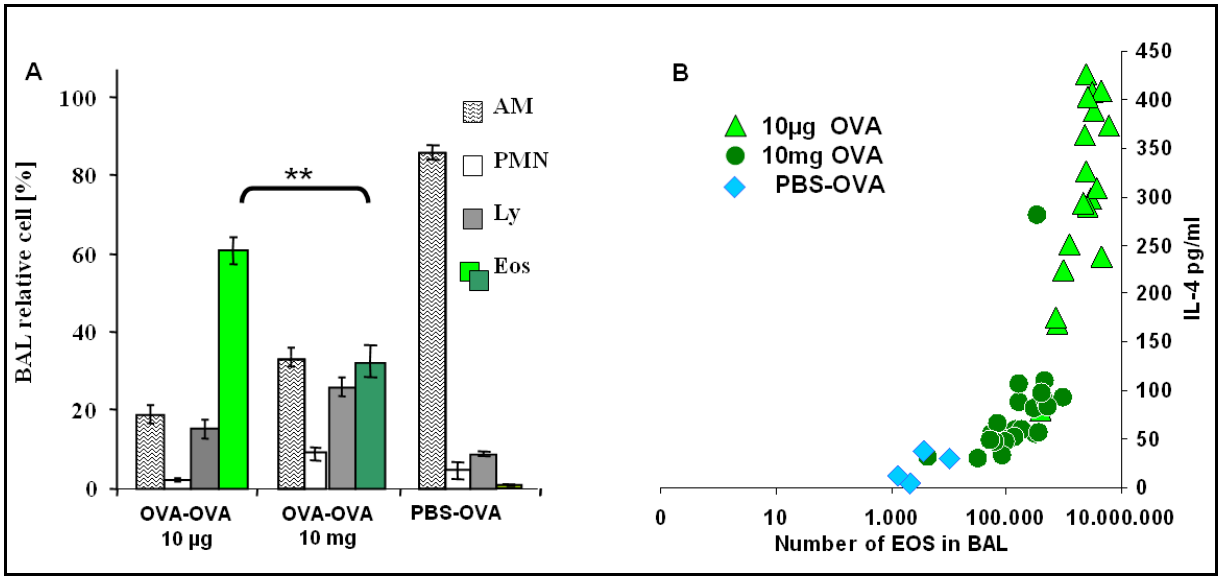


**Fig. 16 Relative number of type of cells in BAL and Eosinophils vs. IL-4 in BAL**

A) Broncho-alveolar lavage (BAL) was analyzed with regard to the relative number of AM, alveolar macrophage; PMN, polymorphonuclear granulocyte (neutrophils); Ly, lymphocyte; Eos, eosinophils. Significant differences in the relative number of eosinophils between Low (10µg-OVA) and High (10mg-OVA) can be observed. PBS-OVA control animals show almost no eosinophils. B) Eosinophils vs. IL-4 in BAL. The values of BAL eosinophilia were correlated to IL-4 production in the BAL fluid. It shows that IL-4 and the amount of eosinophils in BAL are positively correlated and it demonstrates a clear distinction between the two groups low and high dose. Comparative analysis of the allergic phenotype induced by the low dose OVA protocol or the high dose OVA protocol, using a sensitization dose of 10 µg or 10 mg OVA, respectively. Mice were sensitized i.p. -OVA and exposed to OVA-aerosol in a long protocol. Specimens were analyzed 24 h after the last challenge. As a control, naïve mice were sensitized with PBS followed by OVA-aerosol challenge. All groups analyzed consisted of at least 5 animals.

## 6.2 Detection and phenotype of allergen-specific CD8+ H2-Kb SIINFEKL+ T cells

Allergen-specific CD8+ H2-Kb SIINFEKL+ T cells in peripheral blood, BAL and lungs of sensitised mice were found 24 h after the last aerosol challenge, whereas no allergen-specific CD8+ H2-Kb SIINFEKL+ T cells were found in naïve (not sensitised) mice exposed to OVA-aerosol challenge (Fig. 17). The phenotype of the allergen-specific CD8+ T cells infiltrating the lung tissue was characterized. These experiments revealed that in both protocols, the low dose and the high dose OVA model, MHC multimer-positive cells stained CD62L-, CD25+/-, CD44+, CD127+/-, CD69+ and Ly6C +/- as a consequence the majority of cells correspond to activated effector type CD8+ T cells (Ashton-Rickardta and Opferman, 1999; Curtsinger et al., 1998; Huster et al., 2004; Jaakkola et al., 2003; Kaech et al., 2003; Walunas et al., 1995; Wherry et al., 2003). Fig. 18 gives an example of the typical staining pattern obtained with

these phenotypic markers in mice sensitized according to the high dose OVA model. Although no phenotypic difference of allergen-specific CD8+ H2-Kb SIINFEKL+ T cells was brought to light in the two opposing models, significantly higher percentages of CD8+ H2-Kb SIINFEKL+ CD62L- cells were found in the high dose model (Fig. 19).

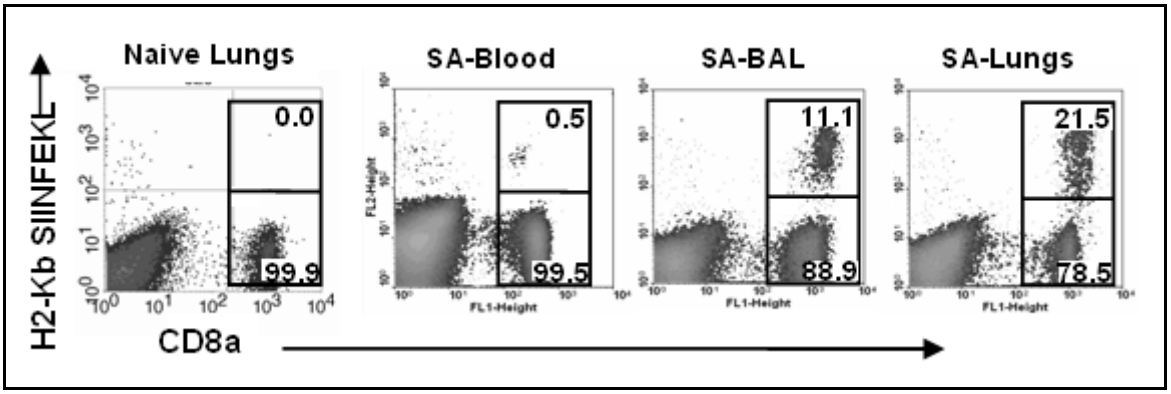


**Fig. 17 H2-Kb SIINFEKL+ CD8a+ T cells in the mouse model of allergic sensitization**

Detection and phenotype of CD8+ H2-Kb SIINFEKL-specific T cells. Mice were sensitized i.p. -OVA and exposed to OVA-aerosol in a long protocol *A*, Percentages of CD8+ H2-Kb SIINFEKL+ T cells among total CD8+ T cells after the final OVA-aerosol challenge in lungs of naïve animals (no OVA sensitization but with OVA-aerosol challenge), or of animals that received OVA sensitization and subsequent OVA-aerosol challenge (SA). CD8+ H2-Kb SIINFEKL+ T cells were analyzed in blood (SA-blood), broncho-alveolar lavage (SA-BAL), and lung tissue (SA-lungs).

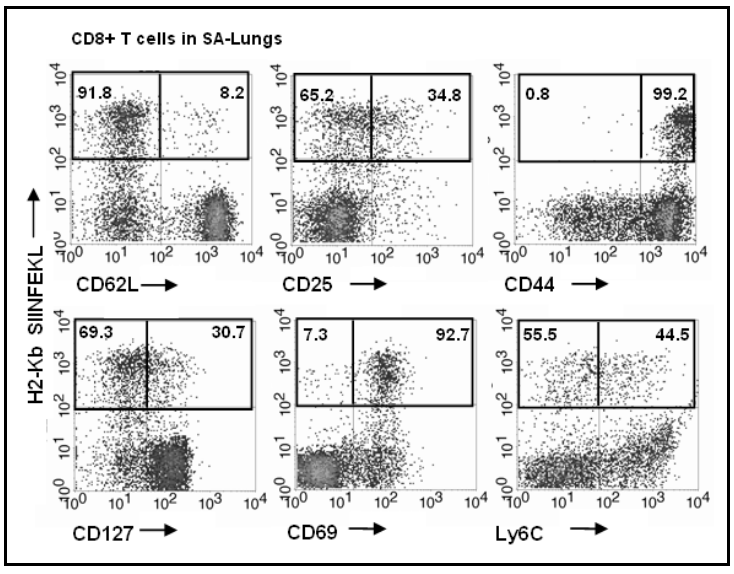


**Fig. 18 Cells phenotypic markers in allergen-specific CD8+ H2-Kb SIINFEKL+ T cells**

Phenotypic surface marker expression of lung CD8+ H2-Kb SIINFEKL+ T cells 24 h after the final OVA-aerosol-challenge. The percentage of cells in each quadrant was calculated by gating CD8+ H2-Kb SIINFEKL+ T cells. Allergen-specific CD8+ H2-Kb SIINFEKL+T cells typically stained negative for CD62L, intermediate for CD25, highly positive for CD44, low to negative for CD127, positive for CD69 and intermediate for Ly6C, thus corresponding to an activated effector CD8+ T cell phenotype.

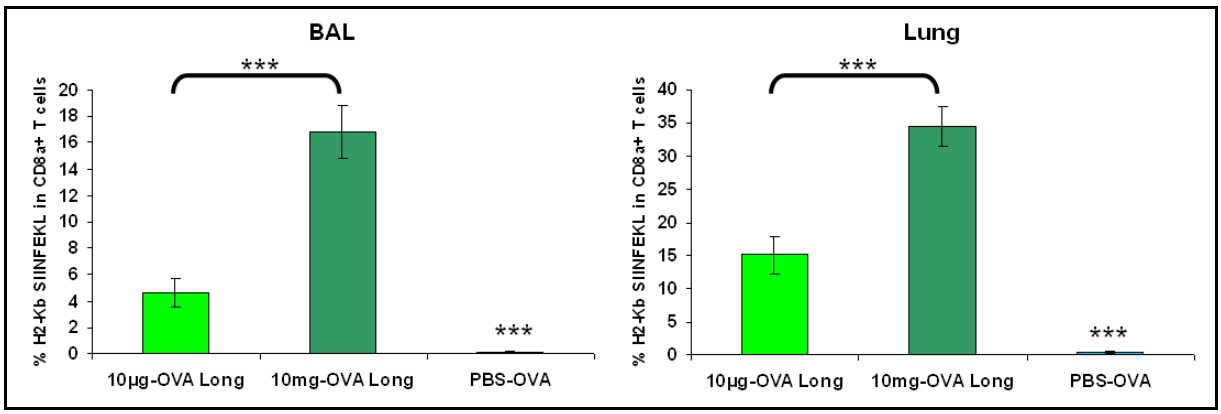


**Fig. 19 Percentages of allergen-specific CD8+ H2-Kb SIINFEKL+ T cells in low and high dose**

Percentages of CD8+ H2-Kb SIINFEKL+ CD62L- T cells among total CD8+ T cells after the final OVA-aerosol-challenge. 10µg OVA low dose protocol (light green), 10mg OVA high dose protocol (dark green). PBS-OVA: naïve mice with just OVA-aerosol-challenge. *=P< 0,05 **=P< 0,01 ***=P< 0,001

Additional experiments in BAL using beads for counting the absolute number of cells indeed confirm that the amount of CD8+ H2-Kb SIINFEKL+ T cells in high dose OVA was significantly higher (Fig. 20 A), although the total number of CD8 + T cells between low and high dose did not change (Fig. 20 B). More evident to realize with the absolute counting technology was that the amount of CD4+ T cells in high dose OVA was reduced which was also reflected in the CD3 compartment (Fig. 20 B).

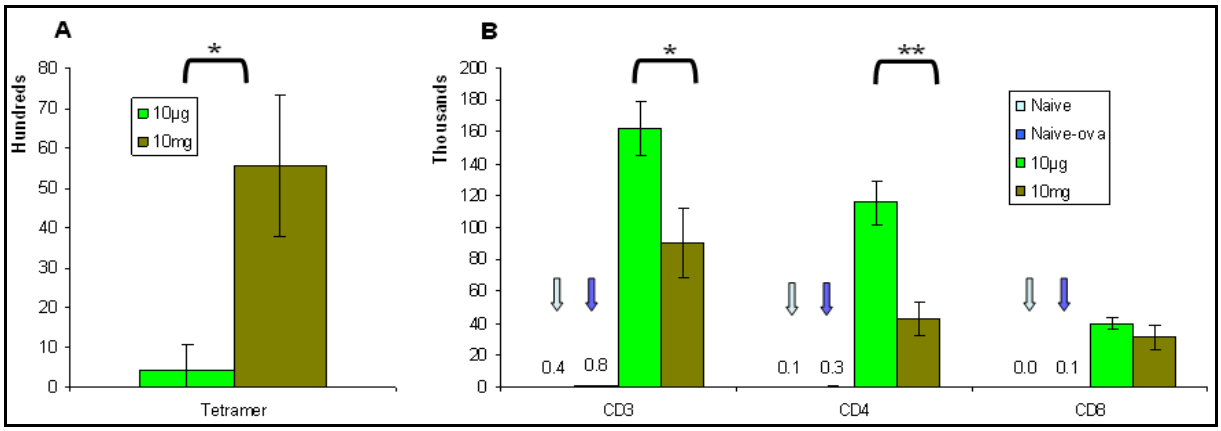


**Fig. 20 Quantification cells in BAL using beads**

Detection and counting of CD3+, CD4+, CD8+ and CD8+ H2-Kb SIINFEKL-specific T cells in , broncho-alveolar lavage (BAL) using beads. Mice were sensitized i.p. with OVA and exposed to OVA-aerosol. A) CD8+ H2-Kb SIINFEKL-specific T cells were significantly different between the two protocols. B) absolute number of cells in BAL, cells are classified as CD3+, CD3+CD4+ and CD3+CD8+, Controls, naïve and naïve-ova (just OVA-aerosol-challenge) animals show very few numbers of cells.
*=P< 0,05. **=P< 0,01. ***=P< 0,001

The decrease of the number of CD4+ T cells in the high dose OVA hints at the fact that the activation of CD4+ T cells raises inflammation. However, to confirm the divergence in the

activation process comparing the two protocols (low and high dose OVA), a direct assessment of CD4+ T Cells and an intracellular staining of CD154 expression after the OVA aerosol challenge was performed. This approach offers an opportunity to gain mouse Th cells specific for defined antigens (OVA in this case) (Frentsch et al., 2005).

With the CD154 marker it was possible to observe that CD4+ T cells are not just more abundant when sensitized with low dose OVA model, but the number of activated OVA-specific CD4+ cells is notoriously higher (Fig. 21a). In order to demonstrate that CD4 + T cells in the high dose protocol are not in a state of anergy, an unspecific stimulation with anti-CD3 was performed. (Fig. 21 b). This unspecific stimulation shows that indeed the CD4+ T cells originated in the high dose OVA model were able to be activated, giving evidence that the state of anergy is directly against the OVA epitopes.

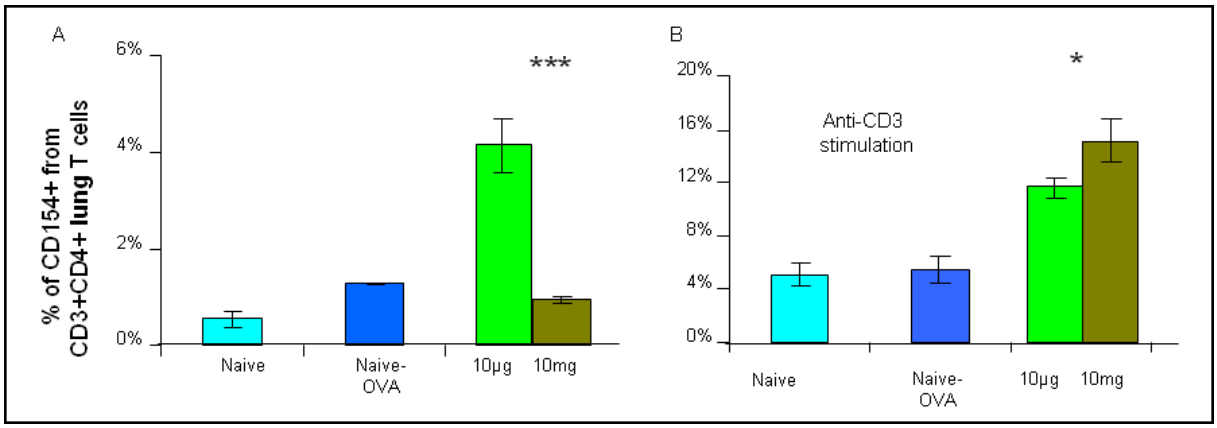


**Fig. 21 Differential CD4+ T cell activation in low and high dose OVA model**

Mice were sensitized i.p. with OVA and exposed to OVA-aerosol, lung cells were harvested and a intracellular staining of CD154 in CD4+ T cells from lung after the final OVA-aerosol-challenge was performed. A) CD4+ T cells directly after the final OVA-aerosol challenge; notice that the low dose OVA protocol (10µg) shows a significantly increased activation pattern in the CD4 T cells when compared to the high dose OVA protocol (10mg). B) CD4+ T cells after 6 h of anti-CD3 stimulation; notice that CD4+ T cells from high dose OVA sensitization are evidently able to produce CD154 in an unspecific way. This indicates that those CD4 + T cells generated in a high dose OVA protocol were not in an anergy state.
*=P< 0,05. **=P< 0,01. ***=P< 0,001

## 6.3 Allergen-specific CD8+ H2-Kb SIINFEKL+ T cell and cytotoxicity

The *in vivo* cytotoxic potential of the induced allergen-specific CD8+ T cells was tested using SIINFEKL-loaded target cells, which were labeled with high concentrations of CFSE and an otherwise identical SIINFEKL-negative target cell population which was labeled with low concentrations of CFSE. After adoptive transfer of the CFSE labeled cells into OVA-

sensitized recipient mice, this *in vivo* approach allowed to quantitate the cytotoxic activity of allergen specific CD8+ T cells by determining the loss of SIINFEKL-positive target cells labeled with high concentrations of CFSE dye (Fig. 9). This experiment clearly showed an in vivo cytotoxic potential of induced OVA-specific CD8+ T cells with a time-dependent mean in vivo killing of 46% (range, 40-50%) at 15 h and 69% (range, 57-80%) at 48 h in the low dose protocol (10µg OVA), and 62% (range, 51-72%) at 15 h and 86% (range, 71-93%) at 48 h in the high dose protocol (10mg OVA) (Fig. 22). At all time points, OVA-specific CD8+ T cells induced in the high dose model displayed a higher cytotoxic capacity as compared to cells induced in the low dose allergy model.

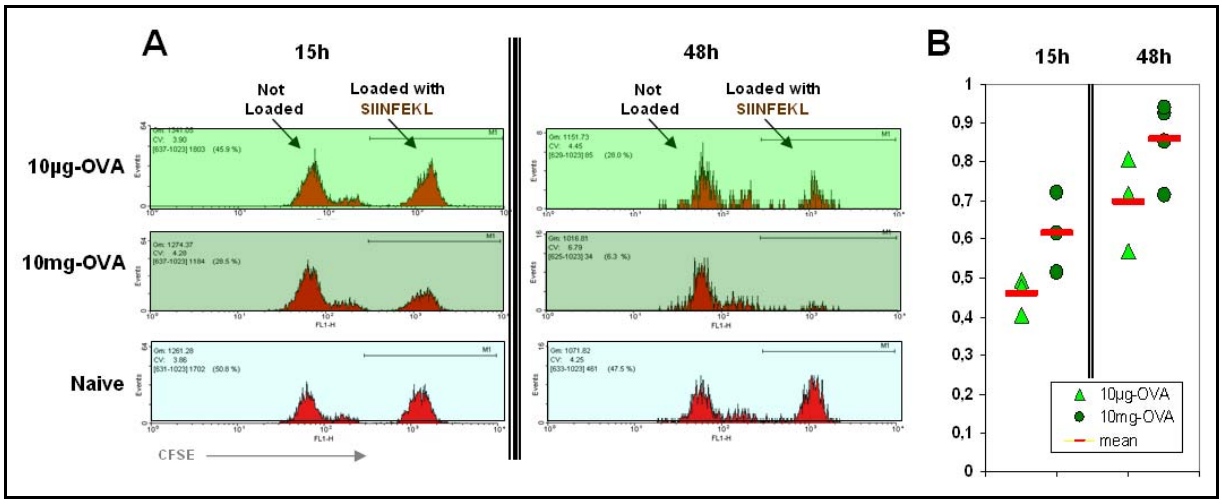


**Fig. 22 In vivo cytotoxicity**

Dependency of the induction of cytotoxicity on the antigen dose used in the sensitization phase.
A) Shows a typical experiment of one mouse of each protocol after 15 h and 48h. B) Time and dose dependency of the *in vivo* cytotoxic potential of allergen-specific CD8+ H2-Kb SIINFEKL+ T cells using splenocytes differentially labeled with CFSE, either SIINFEKL-loaded or -unloaded. Cytotoxicity was recorded by FACS analysis after 0h, 15 h and 48 h. Black dots indicate individual animals sensitized with high dose OVA (10 mg), open triangles animals with low dose OVA (10 µg). The mean of each group is indicated. Mice were sensitized i.p. with OVA on days 1, 10 and 26 and challenged with OVA aerosol on days 82-83, 85-86, 88-89. Cytotoxic potential was demonstrated by in vivo lysis of highly CFSE-labeled SIINFEKL-loaded splenocytes in comparison to unloaded low CFSE-labeled splenocytes.

Additional to the cytotoxicity, the specificity of this CD8+ T cells was confirmed with a supplementary assay of intracellular staining in both naïve and OVA sensitized and aerosol challenge mice. That assay revealed, that when lung cells collected from sensitized and OVA-aerosol-challenged animals are stimulated with OVA-SIINFEKL peptide, the CD8+ T cells synthesized IFNg but no IL-4. (Fig. 23)

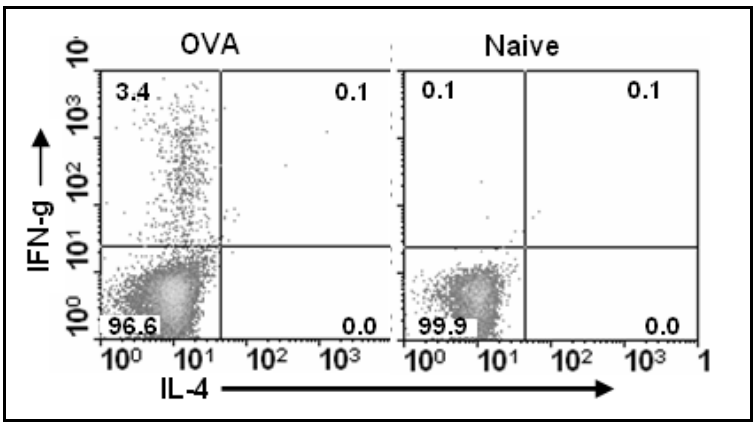


**Fig. 23 Cytokine phenotype of the lung infiltrating allergen specific CD8+ T cells**

Intracellular cytokine profile of lung CD8+ T cells from a representative mouse sensitized i.p. with 10 mg OVA followed by OVA-aerosol-challenge. Cells were collected 24 h after the last airway challenge and were re-stimulated in vitro with 1 µg/ml SIINFEKL peptide for 6h after which intracellular cytokine staining for IFN-g and IL-4 was performed.

## *6.4 Kinetics of allergen-specific CD8+ T cells*

The kinetics and organ distribution of antigen-specific CD8+ T cell generation have been firmly established in infection and vaccination protocols (Klenerman et al., 2002; Knabel et al., 2002), where large numbers of CD8+ cytotoxic T cells are commonly induced in order to provide protection for the host. Therefore, analyzing the natural course of allergen-specific CD8+ T cell induction in the high dose OVA sensitization model is essential.

H2-Kb SIINFEKL multimer staining of allergen-specific CD8+ T cells in PBMCs showed a moderate increase in blood over time ranging from 0.05 to a peak of 0.4% MHC multimer-positive CD8+ T cells among all CD8+ T cells with a clear maximum during allergen re-challenge (up to 0.8%), followed by a contraction of allergen-specific CD8+ T cells immediately after the final OVA-aerosol exposure. Control animals did not show any production of MHC multimer-positive cells at the various time points (Fig. 24 A). The corresponding analysis of lung tissue revealed a dramatic increase of MHC multimer-positive CD62L- CD8+ T cells at the time of the last re-challenge with OVA-aerosol (Fig. 24 B). Interestingly, the number of allergen-specific CD8+ T cells rapidly dropped thereafter but remained stable between 3% and 5% over several weeks, suggesting an organ-specific memory pool of allergen-specific CD8+ T (Fig. 24 B).

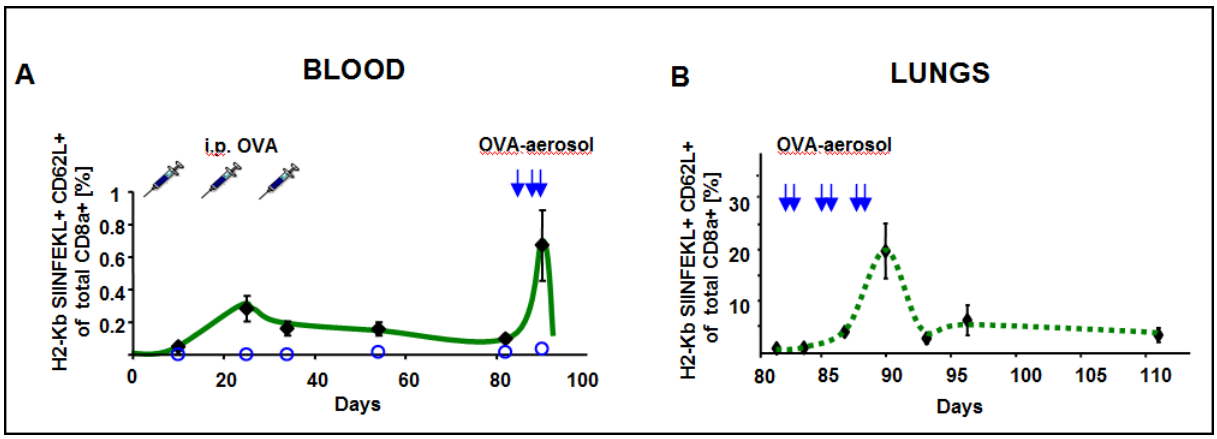


**Fig. 24 Kinetics of allergen-specific CD8+ H2-Kb SIINFEKL+ T cells in blood and lungs**

Kinetics of CD8+ H2-Kb SIINFEKL+T cells in blood and lungs from mice sensitized with high dose OVA (10 mg). OVA-specific CD8+ T cells expand and contract after i.p. application of OVA-alum and after OVA-aerosol challenge as visualized by H2-Kb MHC multimer staining. *A*) Percentages of CD8+ H2-Kb SIINFEKL+ CD62L- T cells among total CD8+ T cells in peripheral blood at different time points. i.p. sensitizations were performed at days 1, 10 and 26, OVA-aerosol challenge at days 82-83, 85-86, 88-89. Filled diamonds represent the group of mice sensitized i.p. with OVA-alum (mean values ± SD, n=5), open circles represent a control group of mice injected with PBS (n=5). *B*) Percentages of CD8+ H2-Kb SIINFEKL+ CD62L- T cells among total CD8+ T cells in lungs at different time points after OVA-aerosol challenge. Arrows indicate time points of OVA-aerosol challenge (days 82-83, 85-86, 88-89).

## *6.5 Organ distribution of allergen-specific CD8+ T cells*

Blood and lung/BAL analyses showed high expansion of MHC multimer-positive CD8+ T cells within the effector organs lung and in the airways as shown by BAL, but only low numbers within the peripheral blood (Fig. 25). In addition to the effector organ lung and the peripheral blood the distribution of allergen-specific CD8+ H2-Kb SIINFEKL+ T cells in several lymphatic organs and the liver were also analyzed to gain information on sites of proliferation and/or activation of these cells. To achieve this, several organs were prepared and the percentage of MHC multimer-positive CD8+ T cells among total CD8+ T cells was determined. As mentioned above, high numbers of activated (CD62L-) CD8+ H2-Kb SIINFEKL+ T cells could be found in lungs and BAL, but also in the liver (up to 10%), whereas bone marrow, spleen, paratracheal, axillary and inguinal lymph node showed percentages below 2% of allergen-specific CD8+ T cells . The lymphatic compartment with the highest number of CD8+ H2-Kb SIINFEKL+ T cells were the mediastinal lymph nodes (2.6%). Of interest, mediastinal lymph nodes also harbored the highest proportion of CD62L+ H2-Kb SIINFEKL+ CD8+ T cells (28%), whereas in all other tissues only a minority of H2-Kb SIINFEKL+ CD8+ T cells was CD62L+ (Fig. 25).

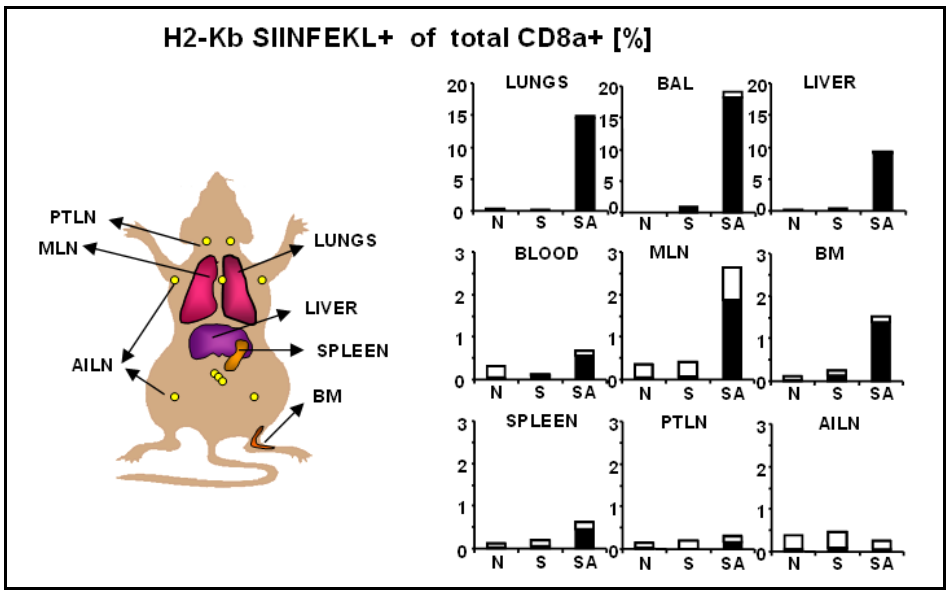


**Fig. 25 Organ distributions of CD8+ H2-Kb SIINFEKL+T cells**

Detection and organ distribution of CD8+ H2-Kb SIINFEKL-specific T cells. Mice were sensitized i.p. -OVA and exposed to OVA-aerosol in a long protocol. Percentages of CD8+ H2-Kb SIINFEKL+ T cells among total CD8+ T cells 24h after the final OVA-aerosol challenge. CD8+ H2-Kb SIINFEKL+ T cells were enumerated in lungs, BAL, liver, blood, mediastinal lymph node (MLN), bone marrow (BM), spleen, paratracheal lymph node (PTLN), axillary/inguinal lymph node (AILN).The filled (black) part of the columns represents the CD62L- fraction (effector cells surface marked) of total CD8+ H2-Kb SIINFEKL+ T cells. N, naïve mouse; S, sensitized mouse; SA, sensitized mouse and challenged with OVA-aerosol.

## 6.6 Recruitment and in situ proliferation of allergen-specific CD8+ T cells

Based on the evidence obtained so far that in the course of sensitization and re-challenge allergen-specific CD8+ T cells are found in increasing numbers in the lungs and the bronchial airways, the question occurred whether this increase was due to an enhanced recruitment of this cell type (which had been proliferating e.g. in lymph-nodes) or if there was proliferation of CD8+ T cells residing in the lungs after allergen exposure. Therefore, purified CD8+ T cells from OT-I mice were labelled with CFSE and transferred into naïve C57/BL6 mice before these chimeras were challenged by OVA-aerosol exposure. This experiment showed that already before allergen challenge there was a population of OT-I cells having migrated to the lungs, most probably due to random migration (Fig. 26 A). After aerosol challenge, however, the cells rapidly started proliferating so that after the third challenge the majority of cells had already undergone several divisions in lung tissue (Fig. 26 B). In contrast, no OT-I cells where detectable in BAL of naïve mice, whereas after OVA-aerosol challenge large numbers of proliferating OT-I cells were found in BAL (Fig. 26 A,B). The result of this experiment argues for both, recruitment into the effector organ and proliferation together with transbronchial migration upon antigen encounter.

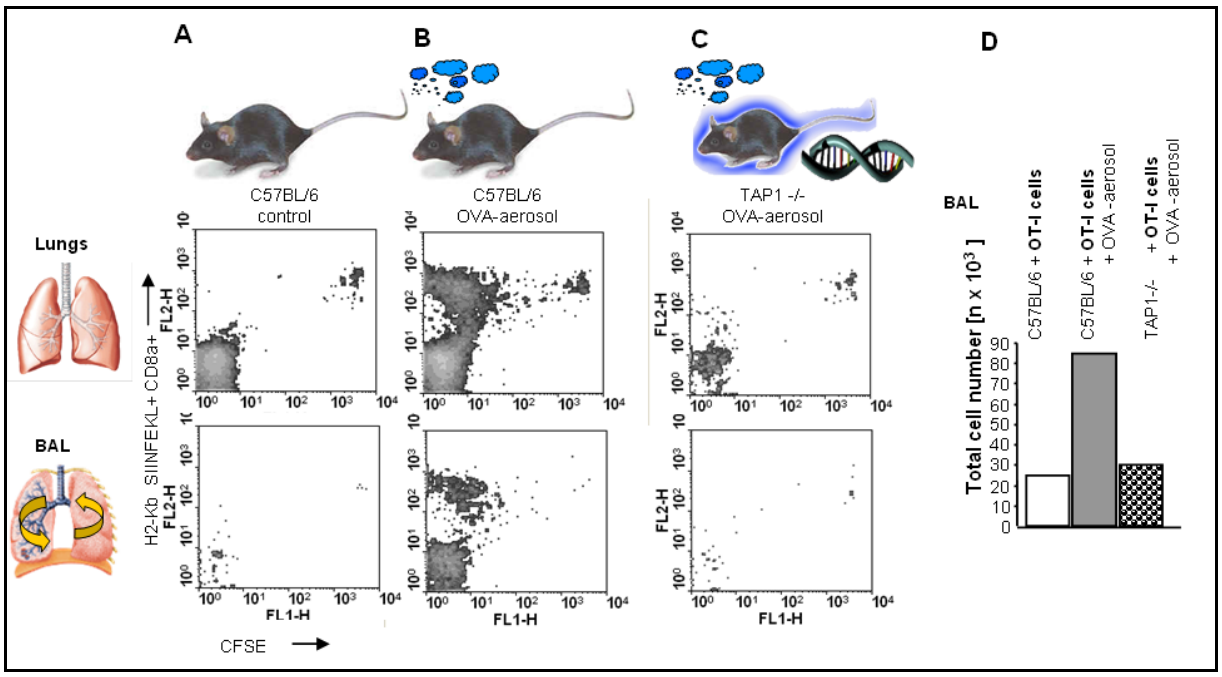


**Fig. 26 Recruitment and in situ proliferation of allergen specific CD8 T cells**

The observed allergen-specific CD8+ cells are induced by cross-presentation. Two groups of naive C57BL/6 mice (n=3 for each group) and a group of transgenic TAP-1 -/- mice (n=3) were adoptively transferred i.v. with CFSE-labeled OT-I cells (~3 x 106 purified CD8+ T cells). One of the C57BL/6 WT groups and the TAP-1 -/- group was exposed to OVA-aerosol challenge. 24 h after the last challenge, animals were sacrificed and cells from lungs (upper panel) and BAL (lower panel) were analyzed by FACS analysis (gated on CD8+ cells). **A)** Naive C57BL/6 mice transferred with CFSE-labeled OT-I cells and challenge with PBS. **B)** Naive C57BL/6 mice transferred with CFSE-labeled OT-I cells and exposed for 1h on three consecutive days with OVA-aerosol (6%, w/v). Notice that almost all cells transferred with CFSE color proliferated and lose their intensity of CFSE. **C)** Naive TAP-1 -/- mice transferred with CFSE-labeled OT-I cells and exposed for 1h on three consecutive days to OVA-aerosol (6%, w/v). Notice that cells transferred did not proliferate. **D)** Total cell number measured in BAL of chimeras adoptively transferred with OT-I cells including a control group of C57BL/6 WT mice that received a PBS challenge, and groups of C57BL/6 WT mice and TAP-1 -/- mice, each challenged with OVA-aerosol (6%, w/v).

## *6.7 Cross-presentation plays a role in allergen-specific CD8+ T cell proliferation*

To test whether cross-presentation plays a role in allergen-specific CD8+ T cell proliferation after OVA-aerosol exposure, TAP1-/- mice have been used on the C57BL/6 background which are deficient in the proper translocation of antigen-derived peptides onto empty MHC-I molecules residing in the endoplasmic reticulum. In an identical set-up as in the proliferation experiments, CFSE-labelled OT-I cells (~ 3 x $10^6$ enriched CD8+ cells) were transferred into TAP1-/- mice and animals were challenged with OVA-aerosol. As shown in Fig. 26 C, OT-I CD8+ T cells migrated into the lungs of TAP1-/- mice but were not capable of proliferating upon OVA-aerosol challenge in contrast to wild-type C57BL/6 mice (Fig. 26 B). Of interest, also the absolute number of inflammatory cells found in BAL was markedly

lower in TAP1-/- OT-I chimeras (n, 2.5 x $10^4$) than in wild-type C57BL/6-OT-I chimeras (n, 8.5 x $10^4$) after OVA-aerosol challenge (Fig. 26 D).

## 6.8 Allergen-specific CD8+ T cells and the pulmonary inflammatory response

As several reports that relied on depletion of CD8+ T cells in the murine model have shown contradictory roles for CD8+ T cells in IgE-mediated allergy (Holmes et al., 1997a; Holmes et al., 1997b; MacAry et al., 1998; Schaller et al., 2005; Stock et al., 2004), we decided to evaluate the natural course of the low dose and the high dose OVA models for allergic sensitization with multiple correlations comparing the generation of CD8+ H2-Kb SIINFEKL+ T cells to markers of activation and airway inflammation. In BAL and in cells infiltrating the lungs, more than 90% of the CD8+ H2-Kb SIINFEKL+ T cells showed the phenotype of activated cells as defined by loss of CD62L (Fig. 18). Consequently, a positive correlation of activated CD8+ T cells (as defined by the loss of CD62L expression) and CD8+ H2-Kb SIINFEKL+ T cells, with higher numbers of CD8+ H2-Kb SIINFEKL+ T cells induced by the high dose protocol (dots) as compared to the low dose group (triangles) (Fig. 27 A) could be observed. Also, the proportion of CD8+ H2-Kb SIINFEKL+ T cells detected in BAL was inversely correlated to the degree of airway eosinophilia, whereby the two dosage groups could be clearly distinguished (Fig. 27 B). Along the same line, the total number of inflammatory cells detected in BAL after OVA-aerosol challenge was negatively correlated with the proportion of infiltrating CD8+ H2-Kb SIINFEKL+ T cells (Fig. 27 C). Thus, the more allergen-specific and activated CD8+ H2-Kb SIINFEKL+ T cells infiltrate to the effector organ lung, the less eosinophilia and total cell infiltration is found in BAL. Again, this analysis resulted in a clear-cut distinction of the two groups receiving either the low dose or the high dose OVA-sensitization protocol.

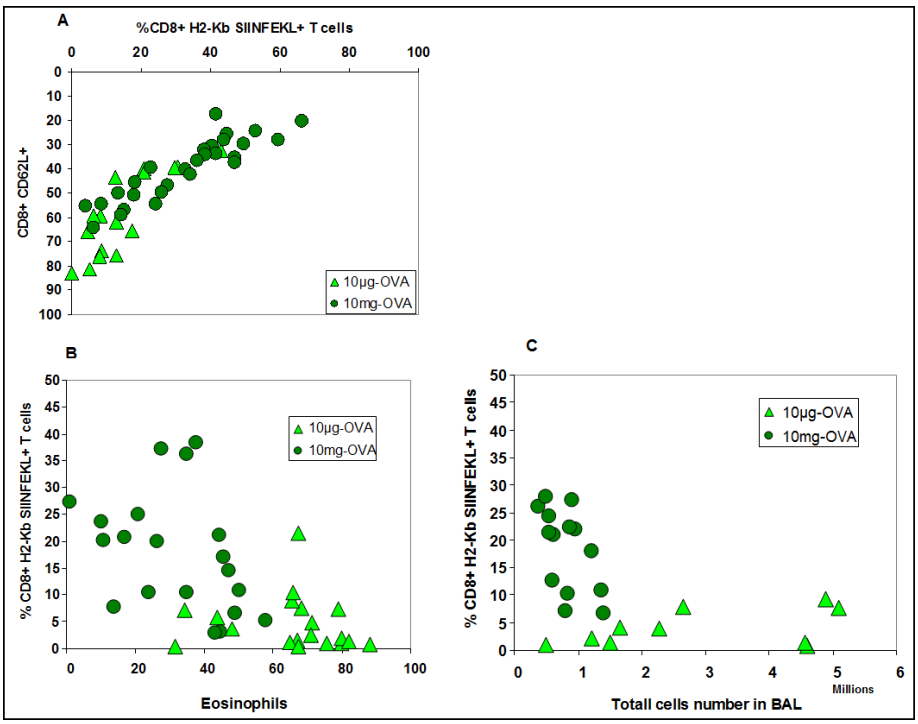


**Fig. 27 Meta-analysis of both low and high dose protocols**

Meta-analysis of the high dose OVA-model (dark dots) and the low dose OVA-model (triangles) of allergic sensitization. Mice were sensitized *i.p.* with 10 µg or 10 mg OVA on days 1, 10 and 26 and challenged with OVA aerosol on days 82-83, 85-86, 88-89. **A)** Correlation analysis of the percentage of the activated CD8+ T cells (CD62L-) and the percentage of CD8+ H2-Kb SIINFEKL+ T cells in lung tissue after final OVA-aerosol-challenge (n=44). **B)** Correlation analysis of the percentage of eosinophils and the percentage of CD8+ H2-Kb SIINFEKL+ T cells in BAL after the final OVA-aerosol-challenge. (n=68). **C)** Correlation analysis of the total number of infiltrated cells in BAL and the percentage of CD8+ H2-Kb SIINFEKL+ T cells in BAL after final OVA-aerosol-challenge. (n=64).

## 6.9 Dose dependent comparison of cytokines between the different allergy models

The process of cellular invasion to inflammatory sites is mediated by the presence of cytokines which are secreted by inflammatory cells upon activation. In order to associate the divergent degree of inflammation upon different sensitizations with the concentration of cytokines in BAL, a titration of the OVA concentration in the sensitization phase was performed using 10 µg, 0.5 mg, 2 mg and 10 mg, in C57BL/6 mice, together with TAP-/- under 10µg OVA sensitization (as a control for cross-presenting pathway deficiency in mice) and an additional control group of C57BL/6 mice with just OVA aerosol challenge without any pre-sensitization.

As already mentioned in section 6.1.1 page 27, when the antigen dose increases in the sensitization phase, the inflammatory response in the lung decreases after the aerosol

exposure; this inverse inflammatory response is also reflected in the amount of cytokines found in BAL (Fig. 28).

The amount of IL-4 and IL-5 present in BAL notoriously varied through the different antigen titrations. Indeed, those cytokines are known to be directly linked to the infiltration of inflammatory cells into the lung (Nakajima and Takatsu, 2007); in addition, IL-10 and IL-12 are also influenced by the titration of antigen, but other cytokines like IL-2, TNF and GM-CSF are affected in a lowered scale (Fig. 28). The amount of eotaxin and IFNg in BAL was below the detection limit.

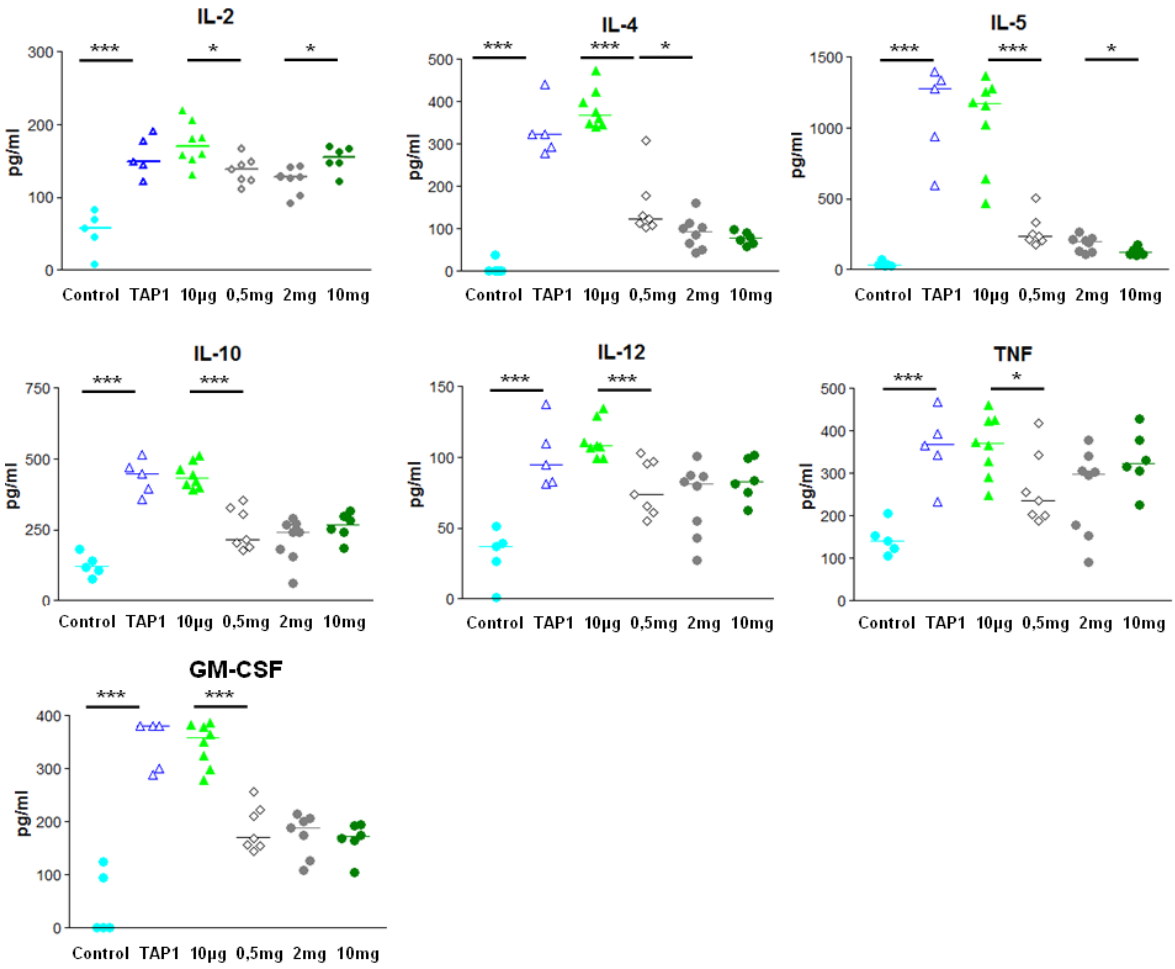


**Fig. 28 Cytokine comparisons between allergy models**

Comparison of airway inflammation and IgE induction using TAP1-/- (10µg OVA) and wild-type C57BL/6 mice in 4 different doses (10µg, 0,5mg, 2mg, 10mg OVA) of allergic sensitization and aerosol challenge. Controls were not sensitized but received OVA aerosol challenge. BAL cytokines were analyzed after allergen challenge with OVA aerosol using Luminex multianalyte system. Mice were sensitized *i.p.* OVA on days 1, 7 and challenged with OVA aerosol (15 min 1% w/v) on days 68-71-74. Specimens were analyzed 24 h after the last challenge (n = 5 to 8 mice per group, *=p< 0.05, **=p< 0.01, ***=p< 0.01).

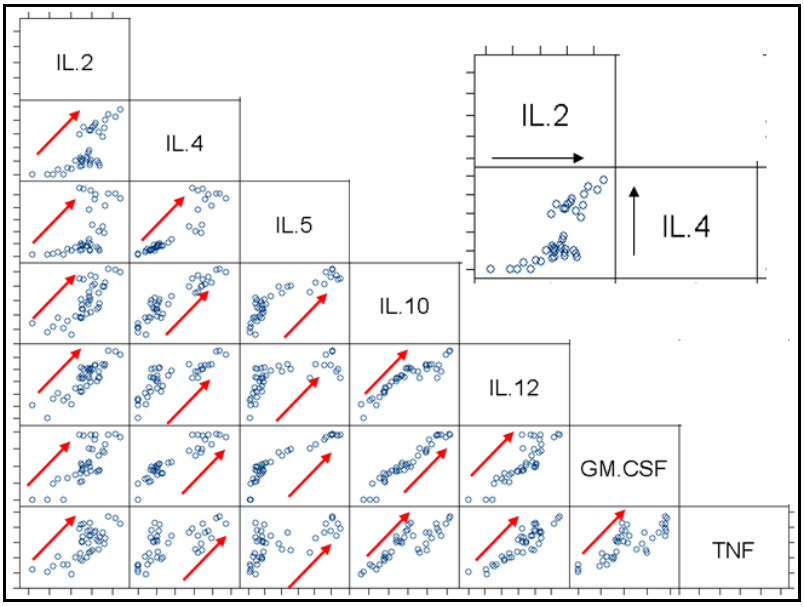


**Fig. 29 Scatter plot matrix of BAL cytokine comparison using accumulative of data**

Cytokines correlation in BAL, displayed in a scatter plot matrix from an accumulative a collection of data from a variety of models. BAL cytokines were analyzed after allergen challenge with OVA aerosol using Luminex multianalyte system.

Resuming the data of cytokine concentration present in BAL from all the different models of sensitization in a scatter plot matrix visualizes that all cytokines measured tend to behave in the same way, whereas there are no cytokines that correlate negatively to the presence of other cytokines. (Fig. 29)

## *6.10 Effect of CD8+ T cell depletion during OVA aerosol challenge*

In preliminary experiments of depletion it could be observed that 42% of the gated CD3+ T cells were CD8+ in the naïve stage of a mouse, but following two *i.p.* injections of anti CD8, the CD8+ T cell were almost absent in the three subsequent days (**Fig. 30**). However, the recovering of CD8+ T cells in the blood was progressive, which was verified on day thirteen after the last *i.p.* injection of anti-CD8 antibody. Moreover, animals that were injected with 200µg contain fewer CD8+ T cells than animals injected with 100µg.

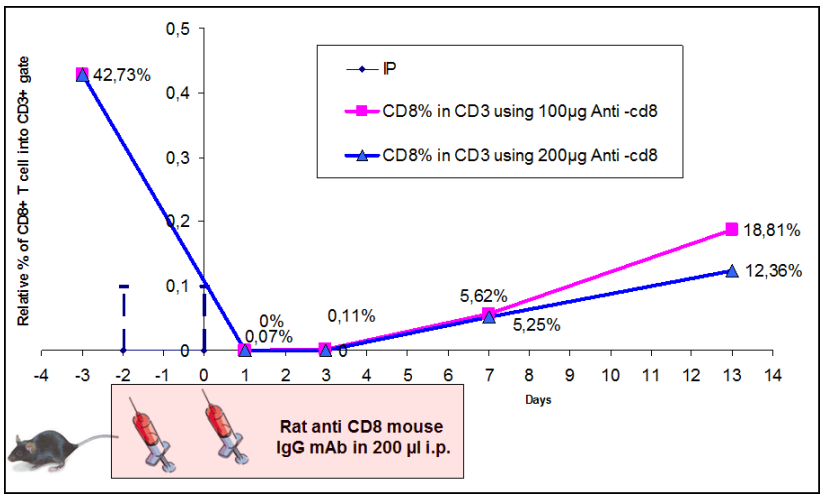


**Fig. 30 The efficiency of CD8+ T cell depletion with only two i.p. injections**

Two groups of WT C57BL/6 mice were *i.p.* injected with anti-CD8 antibody; one group with 100µg and the other with 200µg, both on days -2 and 0. Blood samples were taken on days -3 , 1 , 3, 7 and 13; the cells were analyzed with Flow Cytometry. Staining with CD3+ and CD8+ were performed. The relative percentage of CD8+ T cells in correlation to the CD3+ T cells is shown. After the second *i.p.* injection for depletion, almost no CD8+ T cells were detected in both concentrations (100µg or 200µg); it could be observed that the recovery of CD8+ T cells increased with time. The amount of CD8+ T cells after the third day was not higher than 0,11%, and 10 days later the percentage of CD8+ T cells was 18,81% (100µl and 12,36 (200µg). However, mice injected with 100µg were able to increase the percentages of cells faster than those injected with 200µl, which means that recovering of CD8 T cells in vivo is dose dependent. n=3

These preliminary data demonstrate that the rat anti-mouse CD8 antibody was efficient, and the amount of CD8+ T cells in vivo has a recovery rate which is time dependent. This knowledge about the kinetics of CD8+ cell depletion *in vivo*, made designing a protocol for using anti-CD8+ cells possible, as is described in section 5.9 and Fig. 10. Using 100 µg of rat anti-mouse CD8 two times before the aerosol challenge and a third injection during the challenge is a sufficient amount for depleting CD8+ T cells *in vivo*.

Two groups of mice were sensitized with 10mg OVA on days 1, 10 and 26 and challenged with OVA-aerosol over six days. One group of animals was *i.p.* injected with 100µg of rat anti-CD8 for depletion 72h and 24h before OVA aerosol challenge and on the sixth day after the challenge was initiated (Fig. 10). No difference in either the total cell number or percentage of eosinophils was observed (Fig. 31). However, cytokine levels in BAL measured using bead-based assay (section 5.5 page 22) reveal significantly increased levels of IL-4, IL-10 and IL-12, when CD8+ T cells from sensitized mice were depleted (Fig. 31). Meta-analysis of eosinophils vs. IL-4 in BAL, comparing the two protocols high dose, low dose, and a high dose OVA with depletion of CD8+ T, gives evidence of a clear clustering of mice in their corresponding groups (Fig. 32).

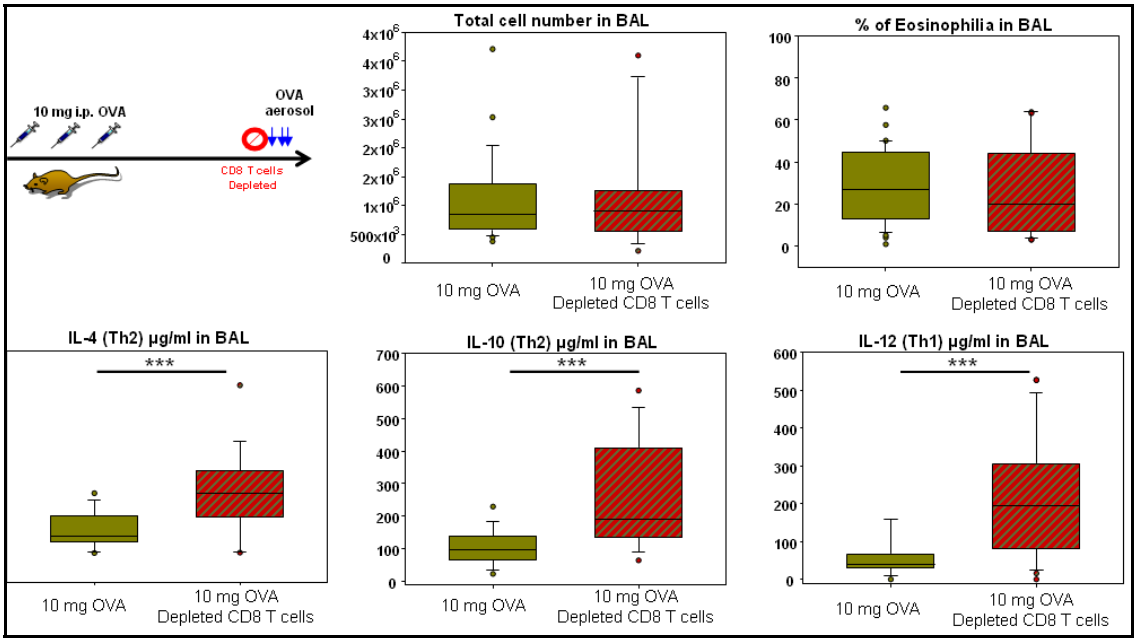


**Fig. 31 CD8+ T cells depletion during OVA-aerosol challenge**

Depletion of CD8+ T cells did not affect the amount of infiltrated cells in the BAL or the eosinophilia values; however, cytokine levels of IL-4, IL-10 and IL-12in BAL were significantly increased. Two groups of mice were sensitized with 10mg OVA on days 1, 10 and 26 and challenged with OVA aerosol, one group of animals was *i.p.* injected with 100µg of rat anti-CD8 for depletion 72h , 24h before OVA aerosol challenge and on the sixth day after the challenge was initiated. (n ≥ 12 per group, data from 4 independent experiments).

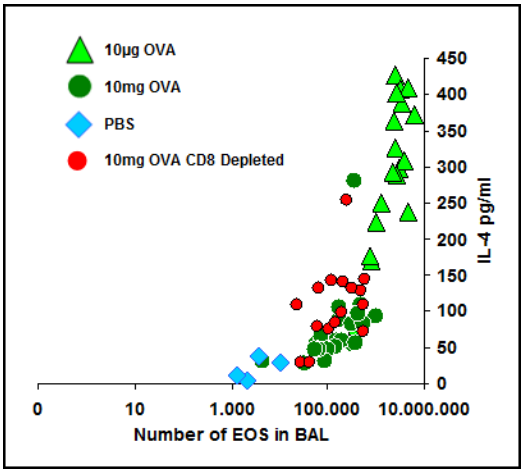


**Fig. 32 Meta-analysis of eosinophils vs. IL-4 in BAL**

Meta-analysis of IL-4 vs. the number of eosinophils found in BAL comparing low dose 10 µg, high dose 10mg and depleted CD8 T cells in high dose group after aerosol challenge. Perceptible clustering of their corresponding groups, and the elevation of IL4 in BAL in the CD8+ T cell depleted mice.

To further investigate the different role of depleting CD8 T cells during the allergen-mediated sensitization, a group was depleted of these cells by systemic application of anti-CD8 mAb either before the sensitization or after the completion of the sensitization and during airway aerosol challenge (Fig. 33 and Fig. 34). As shown in Fig. 33 the depletion of CD8 T cells prior to sensitization (protocol B) resulted in a significant decrease of the total number of cells infiltrated to the BAL. In contrast, depletion of CD8 T cells during the aerosol challenge (protocol C) did not significantly alter the total number of cells infiltrated to the BAL. The relative percentage of cells of the different subtypes of cells found in the BAL reflects the same pattern where the eosinophilia is reduced (Fig. 34). Apart from the cytokines profile described above no changes in either cell distribution or cell number was observed in the depletion during aerosol challenge.

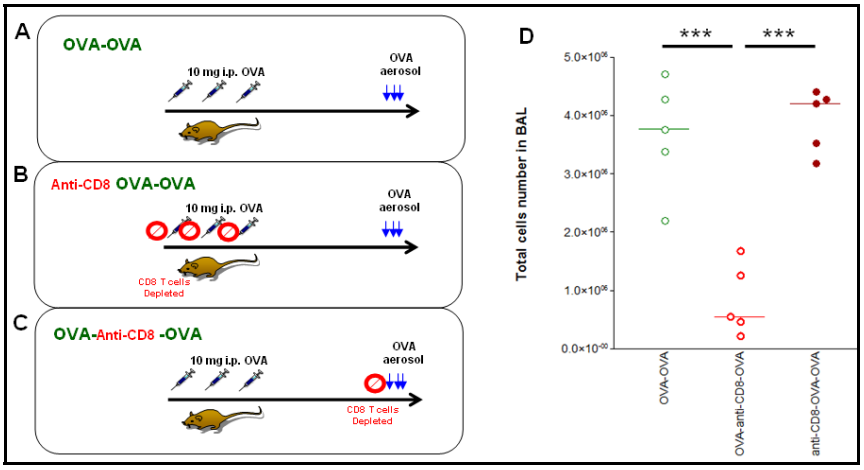


**Fig. 33 Different depletion protocols tested to deplete CD8+ cells**

Anti-CD8 antibodies were used at two different points in time: A) no depletion, B) depletion in the sensitization phase, C) depletion in the challenge phase. D) Total cell number found in BAL after challenge. Mice were sensitized i.p. 10mg-OVA and exposed to OVA-aerosol. Broncho-alveolar lavage (BAL) was analyzed with regard to the relative number of AM, alveolar macrophage; PMN, polymorphonuclear granulocyte (neutrophils); Ly, lymphocyte; Eos, eosinophils

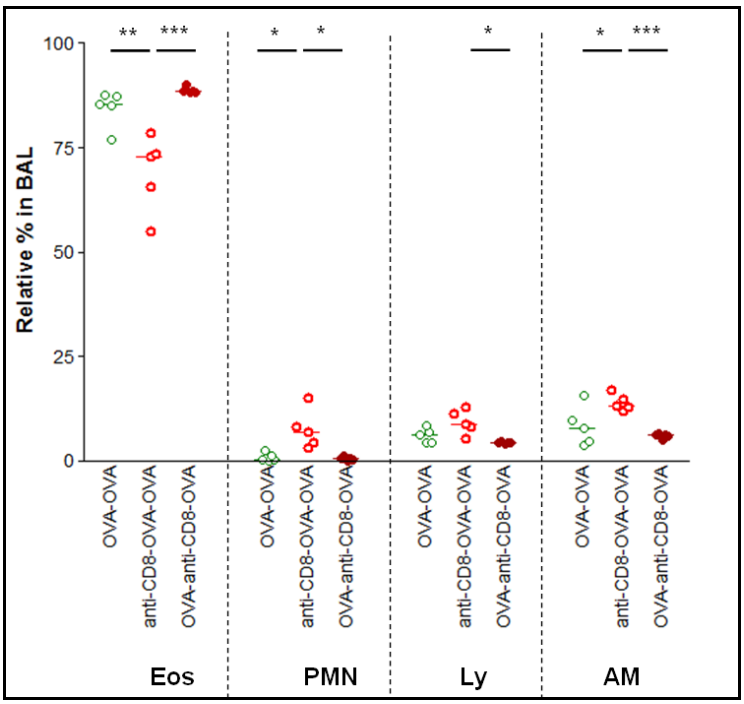


**Fig. 34 Different depletions of CD8+ cells conduce different interpretations**

Analysis of different depletion protocols in the allergic airway inflammation model induced by OVA. Depletion in the sensitization phase lead to a reduced eosinophilia and reduced total cell number infiltrated to the lung, but no difference in cell subtypes or number was observed when anti-CD8 antibodies were used in the challenge phase. OVA-OVA: sensitized and aerosol challenge; anti-CD8 OVA-OVA: CD8 cells were depleted during the sensitizaticion face; OVA-Anti-CD8-OVA CD8 cells were depleted during aerosol challenge. Mice were sensitized i.p. 10mg-OVA and exposed to OVA-aerosol. Broncho-alveolar lavage (BAL) was analyzed with regard to the relative number of AM, alveolar macrophage; PMN, polymorphonuclear granulocyte (neutrophils); Ly, lymphocyte; Eos, eosinophils

## *6.11 Allergy model in Tap-1 -/- mice*

For the present study Tap-1 deficient mice were used in order to observe whether the cytotoxic capacity is crucial or not to develop or decline an allergy phenotype (section 6.3 pag.35). Tap-1 knockout mice are deficient in the cell surface expression of MHC class I molecules, which results in an impaired cross-presentation of antigen and the functionality of the CD8 + T cells is compromised and their number is significantly reduced. (section 5.1, page 17); following this feature, the allergy phenotype might be affected.

TAP1-/- mice were included in the low dose allergen sensitization and challenge model. This model avoids the known limitations of additional depletion of $CD8^+$ dendritic cells as is the case when using anti-CD8 antibodies (Tsuchiya et al., 2009). Using this approach, TAP1-/- mice even showed a significantly higher total number of inflammatory cells infiltrating the

airways compared to wild-type BL6 mice and at the same time, the level of total IgE increased after the allergen challenge. TAP1-/- mice showed a comparably high degree of eosinophilia in the airways (~ 75 %) as compared to wild-type BL6 mice in the low dose OVA protocol (Fig. 35), while compensating for the lack of $CD8^+$ T cells with a higher number of infiltrating $CD4^+$ T cells and $NK1.1^+$ cells .

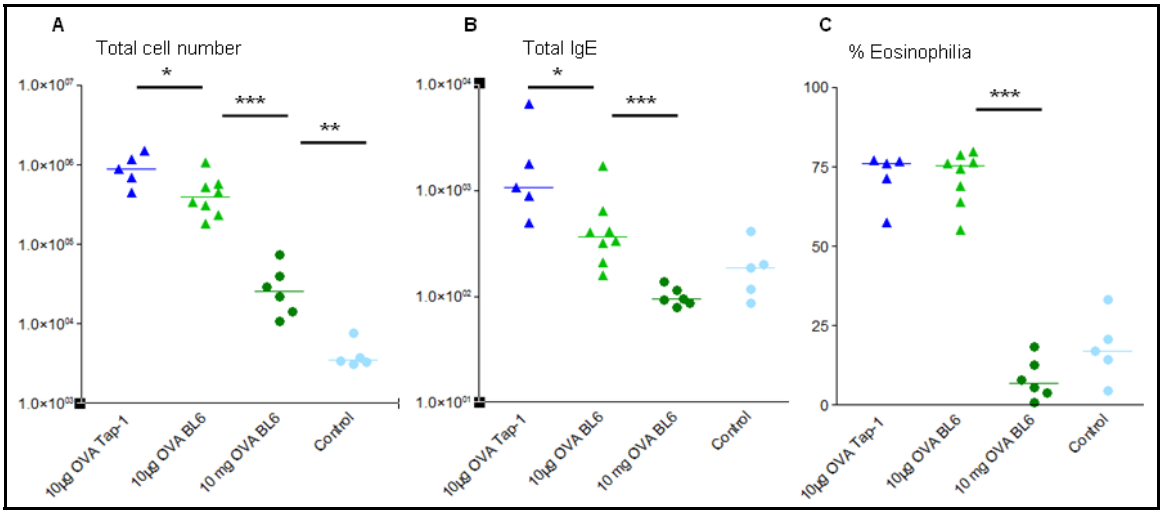


**Fig. 35 Challenge of Tap-1 -/- mice with low dose OVA**

Comparison of airway inflammation and IgE induction using TAP1-/- and wild-type C57BL/6 mice in the low dose (10µg OVA) model of allergic sensitization and aerosol challenge. Controls were not sensitized but received OVA aerosol challenge. Mice were analyzed after allergen challenge with OVA aerosol. (A) shows the degree of allergic airway inflammation as delineated by the total number of infiltrated cells and eosinophilia in BAL (C), as well as the level of total plasma IgE after the allergen challenge (B). Mice were sensitized *i.p.* with 10 µg OVA on days 1, 7 and challenged with OVA aerosol (15 min 1% w/v) on days 68-71-74. Specimens were analyzed 24 h after the last challenge (n = 5 to 8 mice per group, *=p< 0.05, **=p< 0.01, ***=p< 0.01).

# 7 DISCUSSION

Atopic allergic conditions such as asthma, allergic rhinoconjunctivitis, or atopic dermatitis, are characterized by an increase in allergen-specific IgE and $CD4^+$ T cell responses (Kay, 2001). $CD8^+$ T cells which provide protective immunity against viral and bacterial pathogens are considered to be less important (Gavett et al., 1994; Nakajima et al., 1992). However, over the last years, several reports using either gene knock-out strategies or adoptive cell transfer in murine models have indicated a causative role for $CD8^+$ T cells in IgE-mediated allergic inflammation, but their function still is controversial. The present study shows the antigen dose-dependent induction of effector $CD8^+$ T cells with specificity for the soluble antigen OVA in different sensitization protocols. Allergen-specific $CD8^+$ T cells showing a similar phenotype have also been found in humans. Similar to the murine allergy model, the presence of allergen specific $CD8^+$ T cells in patients was correlated to a less severe expression of allergic disease (Seneviratne et al., 2002). Thus, it is reasonable to speculate that $CD8^+$ T cells could play a role in acquiring and/or maintaining a state of clinical tolerance towards an allergenic stimulus in IgE-mediated allergic diseases.

## *7.1 Characterization of a suitable model to study allergic airway inflammation*

As it was disclosed by different titration doses of sensitization (see 6.1, page 27) the allergy phenotype, which is amongst others described as percentage of eosinophils in the BAL together with the total cell number, shows a dose-dependant negative correlation of antigen dose vs. severity of the disease. Other parameters of allergy phenotype, e.g. the IgE levels in plasma (Fig. 12), the inflammatory scoring lung (Fig. 15) and the cytokine profiles (6.9, page 42) also confirm this hypothesis. Although the effect of dose sensitization in the model of allergy has already been described (Morokata et al., 2000; Ohki et al., 2005; Sakai et al., 1999), none of these works, however, illustrated this phenomenon in such a detailed manner, and in addition, no one has pointed out the role of the allergen-specific $CD8^+$ T cells so far. Referring to the lapse between sensitization and aerosol challenge, no major effects in IgE, eosinophilia and total cell count in BAL were observed when comparing long and short protocol (Fig. 12 to Fig. 14). In order to obtain a better picture of the kinetics and the memory phenotype of the allergen-specific $CD8^+$ T cells the use of long period of time between sensitization and challenge can be advantageous. Finally, the natural course of allergen-

specific CD8+ T cells was analyzed with the help of a dichotomist model of murine allergic sensitization and airway inflammation upon opposite antigen dose effects (10 µg, 10 mg OVA).

## 7.2 Detection of allergen-specific CD8$^+$ T cells in the murine model of the allergic airway inflammation

This study describes the successful detection of allergen-specific CD8$^+$ T cells in blood, lung and other organs (Fig. 17 and Fig. 25) with the help of H2-Kb SIINFEKL multimers, which confirms the existence and direct implication of CD8 T cells in allergy in a clear manner.

Those allergen-specific CD8$^+$ T cells were particularly activated and recruited into the lungs of sensitized animals after the allergen aerosol-challenge (Fig. 17 Fig. 24). The pattern, kinetics and distribution of CD8$^+$ T cell activation, expansion and retraction in the course of allergic sensitization and airway inflammation (Fig. 24) strongly resembles the patterns known for viral defense that have been firmly established in infection and vaccination protocols (Klenerman et al., 2002; Knabel et al., 2002) in which a pool of central memory CD8$^+$ T cells persists weeks to months after infection and enters partially in the pool of effector (memory) CD8$^+$ T cells after repetitive antigen encounter (Ashton-Rickardta and Opferman, 1999; Koelle et al., 2002). Interestingly, apart from the effector organ lungs, where antigen is encountered directly by CD8$^+$ T cells after OVA-aerosol allergen challenge, significant numbers of CD8$^+$ H2-Kb SIINFEKL$^+$ T cells were also found in the liver after the allergen challenge (Fig. 18). This implies that a significant proportion of activated CD8$^+$ T cells, after regressing form the lungs, are preferentially retained in the liver as an ultimate site for activated CD8$^+$ effector T cell migration. Yet, the physiological relevance of high numbers of CD8$^+$ H2-Kb SIINFEKL$^+$ T cells within liver tissue after the allergen challenge can not be explained. One contributing factor could be the route of allergic sensitization through i.p. injection of OVA allergen. Furthermore, the clearance of CD8$^+$ H2-Kb SIINFEKL$^+$ T cells from the lungs through draining lymphatics could ultimately lead to the recruitment of allergen-specific CD8$^+$ T cells into the liver, as it has been described in other models of infection or inflammation (Dikopoulos et al., 2003; Masopust et al., 2004; Yoshida et al., 2002).

## 7.3 Phenotype of allergen-specific CD8$^+$ T cells in the murine model of the allergic airway inflammation

No differences in the phenotype of allergen-specific CD8$^+$ T cells induced under both dosage regimens could be found (Fig. 18). However, both, the number of induced allergen-specific CD8$^+$ T cells and the degree of *in vivo* cytotoxicity were significantly higher with the high dose OVA-model when compared to the low dose model (Fig. 18, Fig. 19 and Fig. 22). The expressed phenotype of these allergen-specific CD8 cells after the aerosol-challenge closely resembled the phenotype of activated effector CD8$^+$ T cells with up-regulation of CD69 as early T-cell activation marker, reduced expression of the IL-7 receptor CD127 and almost complete down-regulation of CD62L and high expression of CD44 (Ashton-Rickardta and Opferman, 1999; Curtsinger et al., 1998; Huster et al., 2004; Jaakkola et al., 2003; Wherry et al., 2003). Only the mediastinal lymph nodes harbored a larger proportion of CD8$^+$ T cells expressing CD62L, which indicates a central memory formation (Fig. 25)(Huster et al., 2004).

## 7.4 The mechanism of generation of the allergen-specific CD8$^+$ T cells in the murine model of the allergic airway inflammation

Is cross-presentation the mechanism behind the induction of allergen-specific CD8$^+$ T cells by an extracellular antigen in the challenge phase during the OVA-aerosol?

Various recent publications have defined the phenomenon of cross-presentation. This type of antigen processing describes the association of soluble, extracellular antigens and their respective immunogenic peptides to MHC-I molecules and is dependent on intracellular membrane fusion processes, which allows the passage of soluble antigen peptides into the endoplasmic reticulum where adequate peptides can be loaded onto MHC-I molecules (Ackerman et al., 2003). But not all groups working in that field have agreed to the phenomenon of cross-presentation as a physiological pathway (Zinkernagel, 2002). This question was addressed in this study with the murine model of allergic inflammation by using adoptive transfer of OT-I cells together with TAP1-/- mice in the memory response to soluble OVA-aerosol-challenge (Fig. 26). While strong activation of adoptively transferred CD8$^+$ H2-Kb SIINFEKL$^+$ T cells (OT-I cells) occurred in the lungs of control wild-type C57BL/6 mice after OVA-aerosol-challenge, both in lung tissue and in BAL fluid no activation at all of OT-I cells could be observed in TAP1-/- mice after the allergen exposure. This demonstrates and

argues for the existence of a cross-priming pathway in the OVA allergy model which is operative *in vivo* and is TAP-dependent.

## 7.5 The role of CD8$^+$ T cells in the murine model of allergic airway inflammation can not be understood through depletion experiments

After a successful depletion of $CD8^+$ cells in the challenge phase by the home-made antibodies (Fig. 31) no difference in either total cell number or percentage of eosinophils was observed. Although Th2-prone cytokines in BAL like IL-4 and IL-10 are significantly higher after depletion indicating that the lack of CD8+ T cell will possibly induce an aggravated allergic outcome, at the same time IL-12 Th1-prone cytokine is indicating the opposite. That is why depletion of the CD8+ T cells can not be directly associated to the outcome of the allergic airway inflammation through cytokine interpretation. The failure to clarify the role of $CD8^+$ T cells by using depletion is confirmed by the contradictory data that is shown by others (Holmes et al., 1997a; Holmes et al., 1997b; MacAry et al., 1998; Schaller et al., 2005; Stock et al., 2004). One of the reasons that can describe why depleting CD8 + cells creates confusing interpretation results, can be linked to the fact, that the cluster of differentiation number 8 (CD8) is not just present in T-cells, and then other presenting cells like dendritic cells, who have the same cluster (CD8), can be collaterally depleted. Another reason can be associated with the time point chosen to apply the anti-CD8 antibodies; some additional experiments show that if the depletion occurs in the sensitization face, the outcome of the disease is reduced (Fig. 33 and Fig. 34). That is why the use of transgenic mice like Tap1 deficient mice is better suited than the use of depletion antibodies against CD8 as the repertoire of dendritic cells expressing CD8α is intact in this model.

## 7.6 Specific CD8+ T cells correlate with allergen dose and allergic phenotype

One of the most important findings of this study was the dependency of $CD8^+$ H2-Kb $SIINFEKL^+$ T cell induction and activation on the diverse stimulus doses of antigen in the sensitization phase and the inverse correlation of these cells with the observed allergic phenotype (Fig. 27a). When a low allergen dose of 10 µg OVA was used for allergic sensitization, high titers of total and allergen-specific IgE (Fig. 13) together with substantial pulmonary eosinophilia following OVA-aerosol-challenge were reproducibly obtained (Fig. 14). This model can therefore be considered to be an experimental equivalent to IgE-mediated allergic airway inflammation in humans. In contrast, when a high allergen dose of

10 mg OVA was injected, significantly lower titers of total allergen-specific IgE together with significantly reduced airway eosinophilia following OVA-aerosol-challenge were observed. Compared to the situation in humans, this model most probably represents the situation seen in allergen-specific immunotherapy (Akdis et al., 2006; Gabrielsson et al., 2001). The high dose model, which showed an obvious protective deviation from the Th2 phenotype of the low dose model (Fig. 27 B adn C), was characterized by the induction of significantly higher numbers of activated $CD8^+$ H2-Kb $SIINFEKL^+$ T cells in the effector organ lung and the allergen-exposed airways.

The increase of cytokines in BAL within different models reveals a direct link to the severity of the inflammation; low doses of antigen create a strong inflammation in the lung and consequently produce higher concentrations of cytokines in BAL, whereas with high doses of antigen the severity of the disease is diminished and shows a lower concentration of cytokines. That can be directly linked to the fact that the concentrations of cytokines in BAL are dependent on the number of cells present in this region to synthesize them, and therefore it is possible to speculate that the pattern of cytokines in other tissues, where the number of cells are not directly linked to the amount of cytokines, probably like in lymph nodes, will be different when a different concentration of allergen is applied. An additional deficiency of the antigen cross-presentation has no effect on the cytokine production in BAL, as no changes of cytokines between controls with 10µg OVA (C57BL/6) and the TAP1-/- with 10µg OVA were detected (Fig. 28).

Almost all of the cytokines are known to have a positive impact on promoting the inflammation (see page 44); it is interesting to observe that all plots of correlation have a positive tendency (Fig. 29). This means that the cytokines share the trend to change collectively, which can be explained with the collective synthesis to handle the disease. Additionally, none of the cytokines increases in amount when the inflammatory response is declining.

## *7.7 The role of CD4+ T cells in the different dose models of sensitization*

Of interest, the high number of allergen-specific CD8 cells seems to compensate for a lower number of activated CD4 T-cells in the high dose model and might therefore be a good indicator of protection from type I allergy. A significantly decreased number of CD4+ T cells in the high dose OVA model were corroborated by a lower degree of intracellular activation

marker staining with CD154 in CD4+ T cells (Fig. 21). In addition the staining of BAL CD4+ T cells for Foxp3+ and CD25+ revealed a higher number of Foxp3+ regulatory T cells (Tregs) in the low dose. Furthermore, CD4+ T cells are believed to be the source of other cytokines secreted into the BAL such as IL-13 and IL4, but in the low dose protocol the secretion of this cytokines is up regulated, which may cause a great influence of the CD4+ Tregs dealing with the disease (Aguilar-Pimentel et al., 2009).

## *7.8 CONCLUSION AND PERSPECTIVES*

This study provides new perspectives to the complex question as to whether the role of allergen-specific $CD8^+$ T cells are of beneficial or of detrimental effect on allergy by investigating the antigen dose-dependent induction of $CD8^+$ H2-Kb $SIINFEKL^+$ T cells in the natural course of allergic sensitization and allergen aerosol-challenge in the murine model.

This work argues in favour of a protective role of allergen-specific $CD8^+$ T cells as the number of these cells inversely correlates to phenotype markers of IgE-mediated allergy and airway inflammation such as plasma IgE, airway eosinophilia, cell infiltration and Th2 cytokine levels in BAL. Moreover, using TAP1-/- mice in the low dose model higher degrees of cell infiltration and increased production of IgE as compared to wild-type mice undergoing the same protocol could be found (Fig. 35). For this purpose the TAP1-/- mouse is probably better suited than CD8α-/- or anti-CD8 antibody treated mice as the repertoire of dendritic cells expressing CD8α is intact in this model. Furthermore, the TAP1-/- model targets the induction of OVA-specific $CD8^+$ T cells by way of defective cross-presentation of exogenous antigen (Van Kaer et al., 1992). Even if $CD8^+$ T cell depletion experiments performed in the course of this study did not show a difference either in total cell number or percentage of eosinophils (Fig. 31), animals treated with anti-CD8 antibodies show an increase of cytokines like IL-4 in BAL, suggesting a Th2 profile effect. These results are in agreement with previous findings also using the depletion of $CD8^+$ T cells in models of allergic airway inflammation (Huang et al., 1999; Tsuchiya et al., 2009). The number of allergen specific $CD8^+$ T cells appears to be a critical predictor for the severity of the allergic phenotype in this murine model.

Allergen-specific CD8+ T cells showing a similar phenotype with high production of IFN-g have also been found in humans. Similar to the murine allergy model, the presence of allergen-specific CD8+ T cells in patients was correlated to a less severe expression of

allergic disease (Seneviratne et al., 2002). Thus, it is reasonable to speculate that CD8+ T cells could play a role in acquiring and/or maintaining a state of clinical tolerance toward an allergenic stimulus in IgE-mediated allergic diseases.

This research gave some answers in the context of the role of CD8+ T cells in allergic airway inflammation, but it has also brought about new questions. The implication of CD8+ T cells in killing presenting cells (PCs) has already been demonstrated (section 6.3 page 35), but the enhancement of the CD8+ T cells' potential to kill PCs by using the high dose OVA model (Fig. 22) could not be associated with any cellular marker. Do these cells have an enhanced potential to kill in the particular cellular performance or is the amount of $CD8^+$ H2-Kb $SIINFEKL^+$ T cells responsible for this ability? To resolve the question a refined technique, e.g. the adoptive transfer of effector $CD8^+$ H2-Kb $SIINFEKL^+$ T cells can be applied. Although similar experiments using transgenic mice cells (Stock et al., 2004) have already been performed by others, the adoptive transfer of cells from a WT mice donor which generates effector $CD8^+$ H2-Kb $SIINFEKL^+$ T cells as a natural response to an antigen is still needed. Some preliminary experiments using transgenic listeria monocytogenes which express OVA were performed parallel to this work without delivering any remarkable results (data not shown); in this experiment CD8+ T cells originated in murine infected with transgenic OVA-listeria were adoptively transferred into mice sensitized with OVA; but after OVA aerosol challenge no differences in the severity of the disease could be observed. One reason for the failure of the CD8+ T cells from the listeria model to down-regulate the severity of the allergy can be attributed to the fragility of the cells and the loss of functionality in the new host. Nevertheless, this kind of experiment is to be continued.

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# 10 ABREVIATIONS

| Abbreviation | Meaning |
|---|---|
| Ab | Antibody |
| AHR | Airway hyperresponsiveness |
| AILN | Axillary/inguinal lymph node |
| ALUM | Aluminum hydroxide |
| APCs | Antigen presenting cells |
| BAL | Broncho-alveolar lavage |
| Bio-Plex | ELISA using dyed beads Luminex technology |
| BM | Bone marrow |
| BSA | Bovine serum albumine |
| C57BL/6 | C57 Black 6 , common inbred strain mice |
| CD | Cluster of differentiation |
| CFSE | Carboxy-fluoresceindiacetat succinimidyl ester |
| CTL | Cytotoxic T cell |
| DC | Dendritic cell |
| EDTA | Ethylen diaminetetraacedic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EOS | Eosinophils |
| FACS | Fluorescence activated cell sorting |
| FCS | Fetal calf serum |
| FITC | Fluorescein-isothiocyanat |
| Giemsa | Stain solution containing azure II-eosin, azure II, glycerin, and methanol |
| GMC | German Mouse Clinic |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| H&E, | Hematoxylin and eosin stain solution |
| H2-Kb | One of the major histocompatibility complex I in C57BL/6 |
| high dose OVA | Sensitization with 10mg ovalbumin |
| i.p. | Intraperitoneal |
| i.v. | Intravenously |
| ICH | Immediate Cutaneous hypersensitivity ) |
| IFN | Interferon |
| Low dose OVA | Sensitization with 10µg ovalbumin |
| M | Molar |
| mAb | Monoclonal antibody |
| MHC | Major histocompatibility complex |
| MLN | Mediastinal lymph node |
| OT-I | The transgenic T cell receptor was designed to recognize the immunodominant ovalbumin peptide SIINFEKL (OVA257-264) |
| OVA | Ovalbumin |
| OVA-OVA | Mice sensitized and challenged with OVA |
| PAS | periodic acid Schiff stain solution |
| PBS-OVA | Mice not sensitized and challenged with OVA |

| | |
|---|---|
| PBS-PBS | Mice not sensitized and challenged with PBS |
| PE | Phycoerythrin |
| PTLN | paratracheal lymph node |
| RPMI | Roswell Park Memorial Institute (medium) |
| RT | Room temperature |
| SA | sensitization and subsequent OVA-aerosol challenge |
| SD | Standard deviation |
| SEM | The standard error of the mean |
| SIINFEKL | Ovalbumin peptide (257-264) |
| TAP1-/- | mice are defective in the stable assembly and intracellular transport of class I molecules |
| TCR | T cell receptor |
| Tetramer | H2-Kb SIINFEKL-multimers |
| Th | T helper |
| Th1 | T cell helper (CD4) 1 |
| Th2 | T cell helper (CD4) 2 |
| WT | Wild type |

# 11 Curriculum Vitae

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| | |
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*Allergo Journal (DGAI) 13, 45*

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**28th Congress of the European Academy of Allergy and Clinical Immunology**
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**7th Congreso Internacional Asma-Alergia-Inmunologia**
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**27th Congress of the European Academy of Allergy and Clinical Immunology**
Barcelona, Spain, June 7 - 11, 2008

**4th Central European Bio-Plex® User's Meeting**
Berlin, Germany, Mai 28, 2008

**19th World Allergy Congress**
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**61st AAAAI American Academy of Allergy, Asthma & Immunology**
San Antonio, USA. March 18-22, 2005.

**3rd EAACI-Davos Meeting, Basic Immunology in Allergy and Clinical Immunology**
Davos, Switzerland. February 3-6, 2005.

**16th (DGAI) Mainzer Allergie-Workschop**
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**2nd Annual Meeting of NGFN-Plus and NGFN-Transfer in the Program of Medical Genome Research**
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Munich, Germany. January 26-31, 2009.

**26th Collegium Internationale Allergologicum (CIA)**
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**32nd Arbeitsgemeinschft Dermatologische Forschung (ADF)**
Innsbruck, Austria. March 3-5, 2005.

**25th Collegium Internationale Allergologicum (CIA)**
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**Informa Life Sciences'2nd Annual: Animal Models for Therapeutic Research,**
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## ADDITIONAL CONGRESSES ATTENDED

**EAACI-22 Congress of the European Academy of Allergy and Clinical Immunology (EAACI)**,
Paris, France. 7-11 June, 2003.

**Annual Meeting of the German Society of Immunology (DGFI 34th)**
Berlin, Germany. September 24.-27, 2003.

## MEMBERSHIPS OF SCIENTIFIC SOCIETIES:

**EAACI European Academy of Allergy and Clinical Immunology**