Lehrstuhl für molekulare Allergologie

Sustained Inhibition of the IL-4/IL-13 Receptor Complex during Allergen-Specific Immunotherapy

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This thesis is dedicated to my parents

Contents

List of Figures

List of Tables

Acronyms

γ_c	Common-γ chain
E. coli	*Escherichia coli*
A	Ampere
AIT	Allergen-specific immunotherapy
Alpha-gal	Galactose-alpha-1,3-galactose
Alum	Aluminum hydroxide
AP	Alkaline Phosphatase
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
CD	Cluster of differentiation
CHO	Chines hamster ovary
CTLL2	Cytotoxic T lymphocyte cell line
CysLT1	Cysteinyl leukotriene-1
Da	Dalton
DHFR	Dihydrofolate reductase
ELISA	Enzyme-linked immunosorbent assay
FcϵRI	Fc epsilon receptor I

FcγRIIB	Fcγ receptor IIB
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
G	g force
g	Gram
GATA	Trans-acting T-cell-specific transcription factor
GFP	Green fluorescent protein
h	Hour
HDM	House dust mite
HEK	Human embryonic kidney
i.n.	Intranasal
i.p.	Intraperitoneal
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IFN-γ	Interferonγ
Ig	Immunoglobulin
IL	Interleukin
IL-13Rα-1	Interleukin-13 receptorα-1
IL-13Rα-2	Interleukin-13 receptorα-2
IL-4R-α	IL-4-receptor-α chain
ILC2	Innate type 2 lymphocyte

IRES	Internal ribosomal entry site
JAK	Janus kinase
k	Kilo
KC/GRO	Keratinocyte chemoattractant/human growth-regulated oncogene
L	Liter
LB	Lysogeny broth
m	Milli
MHC	Major histocompatibility complex
min	Minute
Mtx	Methotrexate
n	Nano
OVA	Ovalbumin from chicken egg white
p	Pico
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential hydrogen
PVDF	Polyvinylidenfluorid
RFP	Red fluorescent protein
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
s.c.	Subcutaneous

STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TH	T helper cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic lymphal stromapoetin
V	Volt
°C	Degree Celsius
µ	Micro

1

Abstract

For more than 100 years allergen-specific immunotherapy (AIT) has represented the only curative treatment for allergic diseases. Depending on the type of allergy, the treatment efficacy of AIT varies strongly. Whereas AIT is highly efficient in patients with certain allergies (e.g. yellow jacket venom-allergic patients), it only improves the symptoms of a limited share of patients affected by other allergies (e.g. house dust mite-allergic patients). The exact mechanisms of a successful desensitization are only incompletely understood. However, many hallmarks of an effective AIT have been identified in human studies and animal experiments. Numerous scientific publications identified the reduction of pathogenic TH2 cells as decisive step for tolerance induction. Other up-to-date studies associated the generation of allergen-specific regulatory T cells as indispensable for a successful therapy. Interestingly, the so called "TH2 key cytokine" IL-4 is known to be decisive for both cell populations. It is well established that IL-4 does not only induce the differentiation of TH2 cells from naive $CD4^+$, but that it also suppresses the polarization of *in vitro*-generated Tregs. Although several IL-4 receptor targeting approaches could be shown to be highly efficient in the symptomatic treatment of certain atopic diseases, not much is known about the effect of IL-4 receptor signal inhibition during AIT.

In this study, a potential therapeutic benefit of the combination of AIT with IL-4 receptor inhibition was analyzed. For this reason, an IL-4 receptor antagonist (IL-4 Mutein) was recombinantly produced in a mammalian cell line and purified using affinity- and size exclusion-chromatography. The effect of IL-4 Mutein on TH2- and Treg-differentiation was confirmed in several *in vitro* assays. The IC_{50} value ol IL-4

Mutein could be exactly quantified using a cytotoxic T cell line. Further, its functionality was assessed *in vivo*. Prophylactic IL-4 Mutein treatment inhibited alum-mediated sensitization to the model allergen OVA and house dust mite-induced lung inflammation could be suppressed by IL-4 Mutein administration. Additionally, a PLGA-PEG-PLGA triblock copolymer-based thermosensitive hydrogel was synthesised and used to increase the short serum half-life of the biological. The combination of AIT with IL-4 Mutein administration in a traditional murine model of AIT only resulted in minor changes in the therapeutic outcome. In order to evaluate the effect of IL-4 receptor inhibition during AIT in mice with increased levels of IL-4 and IL-13 a new mose model was established. In this mouse model, the combined treatment of AIT with IL-4 Mutein resulted in several interesting changes on the level of T helper cells. Especially the recently discovered $ST2^+$ $FoxP3^+$ $CD4^+$ cells and $GATA3^+$ $FoxP3^+$ cells were strongly reduced after the combined therapy. Both cell types have been associated with a TH2-like cytokine profile in previous murine studies. It is free of doubt that the relevance of these populations for allergic diseases in human patients needs further clarification. However, IL-4 receptor inhibition seems to represent a promising and precise tool for the therapeutic targeting of these sub-populations.

2

Zusammenfassung

Seit über 100 Jahren stellt die allergenspezifische Immuntherapie (AIT) die einzige kurative Behandlung allergischer Erkrankungen dar. Abhängig von der Art der Allergie variiert die Wirksamkeit der AIT jedoch stark. Während die AIT bei Patienten mit bestimmten Allergien (z.B. Wespengiftallergie) sehr effizient ist, erweist sie sich bei Patienten, die von anderen Allergien betroffen sind (z. B. Hausstaubmilbenallergie), als weitaus weniger wirksam. Wohingegen die genauen Mechanismen einer erfolgreichen Desensibilisierung bisher nur unvollständig verstanden sind, konnten in Studien am Menschen und in Tierversuchen viele Kennzeichen einer effektiven AIT identifiziert werden. Zahlreiche wissenschaftliche Publikationen kamen zu dem Ergebnis, dass die Reduktion von pathogenen TH2-Zellen der entscheidende Schritt für die Toleranzinduktion ist. In anderen aktuellen Studien wurde die Generierung von allergenspezifischen regulatorischen T-Zellen als unabdingbar für eine erfolgreiche Therapie gesehen. Interessanterweise ist bekannt, dass das sogenannte "TH2-Schlüssel-Zytokin" IL-4 für die Differenzierung beider Zellpopulationen von großer Bedeutung ist. Es ist bekannt, dass IL-4 nicht nur die Differenzierung von TH2-Zellen aus naiven $CD4^+$ T-Zellen induziert, sondern auch die Polarisierung von *in vitro* erzeugten Tregs unterdrückt. Obwohl klinische Studien zeigen konnten, dass verschiedene IL-4-Rezeptor-inhibierende Therapien bei der symptomatischen Behandlung bestimmter atopischer Erkrankungen hocheffizient sind, ist nicht viel über die Wirkung der IL-4-Rezeptorsignalinhibierung während der AIT bekannt.

In dieser Studie wurde ein möglicher therapeutischer Nutzen der Kombination von AIT mit der Unterdrückung des IL-4-Rezeptorsignalwegs analysiert. Aus diesem Grund

wurde ein biologischer IL-4-Rezeptorantagonist (IL-4 Mutein) rekombinant in einer Säugerzelllinie hergestellt und unter Verwendung von Affinitäts- und Größenausschluss-Chromatographie gereinigt. Die Wirkung von IL-4 Mutein auf die TH2- und Treg-Differenzierung wurde in mehreren *in vitro* Assays bestätigt. Der IC_{50} Wert des IL-4-Muteins konnte mit einer zytotoxischen T-Zelllinie genau quantifiziert werden. Des Weiteren wurde seine Funktionalität *in vivo* beurteilt. Die prophylaktische IL-4-Mutein Behandlung hemmte die Alum-vermittelte Sensibilisierung gegenüber dem Modellallergen OVA und die durch Hausstaubmilbenextrakt induzierte allergische Lungenentzündung konnte durch IL-4 Mutein Verabreichung unterdrückt werden. Zusätzlich wurde ein thermosensitives Hydrogel auf PLGA-PEG-PLGA-Triblock Copolymerbasis synthetisiert und verwendet, um die kurze Serumhalbwertszeit von IL-4 Mutein zu erhöhen. Die Kombination von AIT mit IL-4-Mutein-Verabreichung in einem traditionellen murinen AIT-Modell führte nur zu geringfügigen Veränderungen des therapeutischen Ergebnisses. Um die Wirkung der Hemmung des IL-4-Rezeptors während der AIT bei Mäusen mit erhöhten Spiegeln von IL-4 und IL-13 zu bewerten, wurde ein neues Mausemodell etabliert. In diesem Mausmodell führte die kombinierte Behandlung von AIT mit IL-4-Mutein zu mehreren interessanten Veränderungen auf der Ebene von T-Helferzellen. Insbesondere die kürzlich entdeckten $ST2^+$ $FoxP3^+$ $CD4^+$ -Zellen und $GATA3^+$ $FoxP3^+$ $CD4^+$ Zellen waren nach der kombinierten Therapie stark reduziert. Beide Zelltypen wurden in kürzlich veröffentlichten murinen Studien mit einem TH2-ähnlichen Zytokinprofil assoziiert. Es besteht kein Zweifel daran, dass weitere Studien nötig sind, um die Relevanz dieser Populationen für allergische Erkrankungen bei menschlichen Patienten genauer bestimmen zu können. Unabhängig davon scheint die Hemmung des IL-4-Rezeptors jedoch ein vielversprechendes und präzises Werkzeug für das therapeutische Targeting dieser Subpopulationen zu sein.

3

Introduction

3.1 Clinical Relevance of Allergic Diseases

In 1906 Clemens von Pirquet introduced the term allergy into the medical society. Originally, he used the term to describe his observation that the body is able to change its reactivity regarding time, quality and quantity (Huber et al., 2004) in a general manner. Today, the term allergy describes the disease following the body´s inappropriate immune response to otherwise harmless environmental substances, called allergens. The term "Atopy", on the contrary, was coined by Arthur Coca and Robert Cooke in 1923 and was used to describe an abnormal type of hypersensitivity against environmental substances (Coca and Robert A. Cooke, 1923). Meanwhile the term is used to describe a subject´s genetic tendency to develop allergic diseases.

The prevalence of allergic diseases has increased dramatically during the last decades. In Western societies roughly 25% of the general population suffers from allergic conditions (Galli et al., 2008). However, allergies do not only affect people in the first world. By now, allergy linked maladies also pose an increasing problem in developing countries (Asher et al., 2006). Today, more than 300 million children suffer from asthma worldwide and there is no end in sight to this trend (Anandan et al., 2010). Numerous scientists even denominate this rapid increase of allergic diseases an epidemic (Eder et al., 2006).

Alongside with allergic asthma, many other allergic diseases like atopic eczema, hay fever or chronic urticaria are on the rise. Even though these diseases affect different tissues and are characterized by different symptoms, they are closely related. A process

that highlights the analogy of these different diseases is the so called "atopic march". This term describes the typical sequence of IgE responses during the life of atopic patients that can go along with different clinical manifestations (Spergel and Paller, 2003). In the clinic, however, the term is regularly used to describe the progression of atopic diseases from atopic eczema to allergic asthma that occurs preferentially in children (Zheng et al., 2011).

Despite the fact that the term allergy is mainly used to describe IgE-mediated diseases, there are other IgE-independent forms of allergic reactions. In the daily clinical routine allergic reactions are classified into four types by a classification system that was established by Coombs and Gell in 1963 (Gell and Coombs, 1963). Although this classification is controversially discussed by today´s clinicians and scientists, it gives a good overview over the different types of hypersensitivity reactions: The so called type I reactions are hypersensitivity reactions of the immediate type and are characterized by IgE-mediated mast cell activation. Allergic rhinitis, allergic asthma, atopic eczema and systemic anaphylaxis are all type I hypersensitivity reactions. Type II reactions describe IgG-mediated antibody responses against cell surface or matrix antigens. Type III reactions are also mediated by IgG antibodies. In this case, the immune response is directed against soluble antigens. Both, type II and type III reactions can result in complement activation and phagocytic effector mechanisms. Type IV hypersensitivity reactions are T cell-mediated. They can be subdivided into T helper cell (TH) 1, TH2 and cytotoxic T cell-dominated hypersensitivity reactions (Janeway CA. Travers P. Walport M. Shlomchik, 2012).

The better insight into these unwanted immune responses is crucial for today´s therapeutic approaches in the clinic. However, due to the diverse mechanisms underlying allergic diseases, a prominent amount of patients still only receives unsatisfactory therapies. In view of the complex heterogeneity of allergic diseases and the yet rising incidences, a better understanding of the dysregulated immune responses is decisive for satisfactory treatments of the majority of patients in the future.

3.2 Causes of Allergic Diseases

Many factors have been identified as risk factors for allergic diseases. One well established determining factor for the development of allergies is the genetic background of

the affected people. Within the last decades, numerous clinical studies have focused on the genetics of allergic diseases. The vast majority of them concluded that the genetic background significantly influences the risk of developing allergic diseases and, furthermore, that allergies are characterized by a strong heritability (Los et al., 2001). However, due to the large variations between the studies, a quantitative estimation of the genetic effect on allergies cannot be made. The calculated heritabilities in the different studies varied for asthma between 35% and 95%, for allergic rhinitis between 33% and 91% and for atopic eczema between 71% and 84% (Ober and Yao, 2011). Also numerous twin studies resulted in significant, but again varying, concordances for allergic diseases in monozygotic twin pairs (Edfors-Lubs, 1971) (Sicherer et al., 2000), (Duffy et al., 1990).

More than 100 genetic loci have been associated with an increased risk for asthma and allergic phenotypes (Ober and Yao, 2011) (Vercelli, 2008). As expected, genes that appear to be particularly linked to an increased risk of allergic diseases include TH2 associated genes like Interleukin-4 (Imboden et al., 2006), its receptor (Guglielmi et al., 2006) and HLA-associated genes (Munthe-Kaas et al., 2007). Furthermore, especially genes that are related to the function of different epithelial derived cytokines such as thymic lymphal stromapoetin (TSLP), Interleukin-33 and its receptor ST2 (Moffatt et al., 2010) (Torgerson et al., 2011) could be linked to allergic diseases. The importance of a faulty barrier function in the development of allergic diseases was highlighted by Palmer et al. (2006). In their study, which attracted a lot of attention, the group was able to demonstrate that loss of function mutations in the gene for Filaggrin, an epidermal barrier protein, are also highly associated with atopic eczema and asthma.

However, the genetic background alone can not be held accountable for a large proportion of allergic incidences. This does not only become obvious in the light of low concordances of allergic diseases in twin studies but also in the rising trend of allergies worldwide that has occurred within the last decades. A famous explanation for this trend was found by David Strachan in 1989. His well-known "hygiene hypothesis" postulates that the increasingly clean environment in western countries is responsible for the elevated prevalence of atopic diseases (Strachan, 1989). More precisely, he hypothesizes that the reduced incidence of bacterial and parasitic infections in the western society and developing countries is decisive for the rise in allergic diseases. Mechanistically, it is speculated that the numerous measures that have been taken to improve the state of

health of the people in the western world (including introduction of vaccinations, use of antibiotics, access to clean water, food safety laws and a strong decrease in parasitic infections) has resulted in a more sterile environment and a decreased infectious burden of people. In compliance with Strachan´s hypothesis, several studies could draw a link between infectious burden and the incidence of allergic diseases (Janson et al., 2007).

Today, the hygiene hypothesis is widely accepted. Countless epidemiological studies were able to show that the altered environment in western countries correlates with the rise of inflammatory diseases (Okada et al., 2010). Especially, migration studies confirmed the strong impact of the environment on the development of allergic diseases. It could be observed that migrants from countries with low allergy incidences acquire allergic diseases with higher incidence at the first generation (Cabieses et al., 2014) in countries with an increased allergy prevalence. Furthermore, a study by Bach (2002) was able to show a positive correlation between gross national product of a country and its incidence of asthma.

However, the hygiene hypothesis is not only based on epidemiological data. Also numerous animal studies could demonstrate a causal connection between parasitic infections and the susceptibility to allergic diseases. Mechanistically it is difficult to pin down the hygiene hypothesis to one single mode of action. Different studies focus on different consequences of a more sterile environment. One possible explanation can be found in the reciprocal regulation of TH1 and TH2 cells: According to certain studies, a lack of microbial infections results in a down regulation of TH1 cells, which in turn results in a decreased secretion of the TH2-suppressing cytokine interferon-γ (Oyama et al., 2001). Other studies put Toll like receptor (TLR) 4 stimulation in the focus of attention (Velasco et al., 2005). According to these studies, TLR4 agonists, which include microbial products, could decrease TH2-allergic responses. Another theory suggests that a reduction of cells with a regulatory phenotype is responsible for the increased susceptibility to allergic diseases (Belkaid et al., 2002).

A modern extension of the hygiene hypothesis focuses on the microflora of allergic and healthy subjects instead of the incidence of pathogenic infections. This so called "biodiversity hypothesis" puts the diversity of the microflora of the body in the center of attention (Riiser, 2015). Different studies drew a negative correlation between the microbiome´s biodiversity of the gut and the risk of developing asthma (Haahtela et al.,

2013). Of particular importance for this hypothesis is the frivolous use of antibiotics in western societies.

Additionally to these incompletely understood complex mechanisms that could be responsible for the onset of allergic diseases, one well documented distinct cause of allergy was identified by the group around Zhomas A.E. Platts-Mills. In 2008 the group was able to associate the onset of a certain food allergy with previous tick bites (Chung et al., 2008). More precisely, they could demonstrate that bites of the tick *Amblyomma americanum* (Lone star tick) are able to induce high IgE titers against the mammalian oligosaccharide epitope, galactose-alpha-1,3-galactose (alpha-gal) which is found in red meat and in certain recombinantly expressed antibodies (Steinke et al., 2015).

The diverse environmental and genetic causes of allergic diseases illustrate their heterogeneity. A better understanding of our environment and our immune system is necessary in order to counteract the rising trend in allergic diseases and to develop more precise treatment strategies.

3.3 Immunological Mechanisms of Allergic Inflammation

As described in chapter 1.1, hypersensitivity reactions can be subdivided into four different types. However, this chapter will solely focus on the molecular mechanisms of the immediate type I reaction as this TH2-dominated and IgE-mediated immune response is the pathomechanism underlying the most common allergic diseases, such as allergic asthma, hay fever or atopic eczema.

It is well established that the molecular mechanisms of the type I allergic inflammation strongly resemble the inflammation caused by infections with helminths or ectoparasites. Both immune responses are characterized by an increased IgE production and driven by TH2 cells. Simplified, the body´s inflammatory immune response to certain allergens is caused by the immune system´s misconception of an harmless environmental or food protein for a parasite derived compound (Wilson et al., 2005) (Mangan et al., 2004). Hence, it responds with a dysregulated type 2 inflammation mediated by TH2 cytokines, such as Interleukin-4 (IL-4), IL-5, IL-9, and IL-13. The process of allergic immune responses can be divided into different phases: The sensitization to the allergen, the acute inflammation after single allergen exposure and,

potentially, a chronic inflammation following repeated allergen exposure. The following paragraphs aim at elucidating the molecular mechanisms of the different stages of allergic inflammation.

3.3.1 Sensitization

The sensitization to an allergen is the first and crucial step in the development of allergic diseases. Allergic sensitization describes the process in which an allergen comes in contact with the barrier epithelium of an organism and induces a TH2 immune response. The cytokine secretion profile of this response is dominated by the TH2 key cytokines IL-4 and IL-13 that are able to induce the class-switching of B cells and, ultimately, the production of allergen specific IgE. There are numerous factors that determine whether a contact with an allergen results in the organism´s sensitization. Not only the genetic background and the environment of the organism are decisive. A further crucial parameter is the route of allergen entrance. Different routes of sensitization result in different sensitization profiles in a murine model of airway inflammation (Repa et al., 2004). Additionally, the biochemical characteristics of the allergens are important. Many well studied allergens are known to possess protease activity. The targets of the proteolytic activity are very heterogeneous. Some proteolytic allergens activate T cells via cleavage of CD25 (Harris et al., 2004). Others increase the permeability of the barrier epithelium by cleaving tight junction molecules (Wan et al., 1999). Numerous further targets were identified within the last years (Hammad and Lambrecht, 2008). Further on, the presence of adjuvant substances has a decisive effect on the sensitization process. Depending on the dose, Toll-like receptor 4 agonists, such as lipopolysaccharides, can have either protective or enhancing effects on allergic sensitization to allergens (Eisenbarth et al., 2002). A very prominent natural adjuvant substance is chitin. This biopolymer that is found in insects and helminths is a strong inducer of IL-4-expressing innate immune cells (Reese et al., 2007) and, therefore, supports sensitization processes.

Mechanistically, the first step in a sensitization event is always the uptake of the allergen by dendritic cells. This can occur either outside of the epithelial barrier or, following a barrier disruption, in the local tissue. After allergen uptake, DCs migrate to a local secondary lymphoid organ where they present processed peptides via their MHC-II molecules to naive $CD4^+$ T cells. Due to the presence of IL-4 of so far unknown source, these naive antigen specific T cells differentiate into committed TH2

cells. However, IL-4 is not the only important factor involved in the polarization of TH2 cells. Furthermore, the circumstances of allergen contact, which could include tissue damage and release of alarmins, presence of TLR agonists or other factors that activate cells of the innate immune system, also play a role in TH2 differentiation. A well-studied example is the production of TH2 cytokines IL-5 and IL-13 by type 2 innate lymphoid cells (ILC2s) upon activation with IL-25, IL-33 or TSLP, which results in a TH2 supporting local milieu (Gold et al., 2014).

Upon allergen exposure, TH2 cells produce large amounts of TH2 cytokines, such as IL-4, IL-5, IL-9 and IL-13. The secreted IL-4 is indispensable for the class switch of B cells to an IgE-producing phenotype. However, TH2 cells are not only involved in the class switch via their cytokine secretion, but also via their expression of different co-stimulatory molecules, such as CD40L and CD28 that bind to surface molecules on B cells and supply the cells with the necessary co-stimulatory signals (Cerutti et al., 1998), (Lumsden et al., 2003). In the process of class switching, gene segments that are coding for the heavy chain of the immunoglobulin are rearranged so that antibodies of the IgE type are secreted. Subsequently, high amounts of IgE are released into the system, where they bind to the high affinity receptor Fc epsilon receptor I (FcϵRI) on tissue resident mast cells or basophils. This whole sensitization phase is not accompanied by any clinical symptoms. Only further allergen exposure induces the actual allergic inflammation that is described in the following section.

3.3.2 Allergic Inflammation

An allergic reaction is triggered when a sensitized organism encounters its specific allergen. The allergic inflammation can be subdivided into the so called "early phase response" and the "late phase response".

3.3.2.1 Early Phase Response

The early phase response occurs within minutes after allergen contact and is based on the allergen-mediated cross-linking of mast cell- and basophil-bound IgE. Unlike most other antibodies, IgE does not bind to immunoglobulin receptors after binding of its specific antigen. Strikingly, it binds to the FcϵRI receptor in an unbound state. Hence, most IgE is not found in a free state but rather fixed to the surface of FcϵRI bearing cells, such as basophils and mast cells (Stone et al., 2010). If multiple cell

bound IgE molecules bind an allergen with several binding sites, the IgE molecules get cross-linked. The following aggregation of FcϵRI induces an intracellular signaling pathway that is responsible for the release of various substances and factors from mast cells and basophils (Janeway CA. Travers P. Walport M. Shlomchik, 2012).

The factors that are released from mast cells during the early phase include preformed substances that are stored in granules in the cytoplasm. These presynthesized factors include histamine, heparin, carboxypeptidases and tryptases. But also presynthesized cytokines, such as tumor necrosis factor-α (TNF-α) or IL-13 are associated with the granules (Gilfillan and Tkaczyk, 2006). Besides the release of granule derived factors, mast cells are able to synthesize and relase phospholipid mediators like prostaglandin D2 and leukotriene B4 (MacGlashan et al., 1982). The diverse panel of factors that is released from mast cells is responsible for a multitude of symptoms. Amongst others, they include contraction of smooth muscle cells in the lung and an increased mucus production. Furthermore, they can be responsible for vasodilation and an increased vascular permeability. In case of a rapid systemic activation of mast cells, these effects can ultimately result in severe anaphylaxis.

3.3.2.2 Late Phase Response

The so called late phase response peaks approximately 6-9 hours after allergen encounter. This delayed response is mainly mediated by antigen-stimulated TH2 cells and activated mast cells. TH2 cells activate cells of the innate immune system like neutrophils and eosinophils via TH2 cytokines (Janeway CA. Travers P. Walport M. Shlomchik, 2012). Additionally, they affect the mucus production and contraction of epithelial cells via production of IL-13. Besides their ability to rapidly release immune mediators upon IgE cross-linking, mast cells are also involved in the late phase reaction. Via expression of different chemokines and cytokines, mast cells are able to shape the immune response following an allergen encounter in either a pro- or an anti-inflammatory way (Galli et al., 2008). Pro-inflammatory functions of mast cells include the production of pro-inflammatory or TH2 cytokines, such as IL-4, IL-13 and IL-6. Anti-inflammatory functions include the expression of IL-10 which suppresses T-cell proliferation and the expression of pro-inflammatory cytokines (Sayed et al., 2008).

Besides TH2 cells and mast cells, numerous other cell types are involved in this delayed immune response. Basophils, eosinophils, neutrophils and monocytes that are

recruited to the site of inflammation are part of the late phase reaction via expression of pro-inflammatory cytokines and other pro-inflammatory substances. Innate type 2 lymphocytes (ILC2s) could be shown to be involved in the recruitment of eosinophils to the lung via strong IL-5 production (Morita et al., 2016). Besides recruited or tissue resident TH2 cells and, depending on the tissue, other T helper phenotypes, such as TH1, TH17 or Treg can also be found at the site of inflammation (Hansen et al., 1999) (McKinley et al., 2008) (Hawrylowicz, 2005).

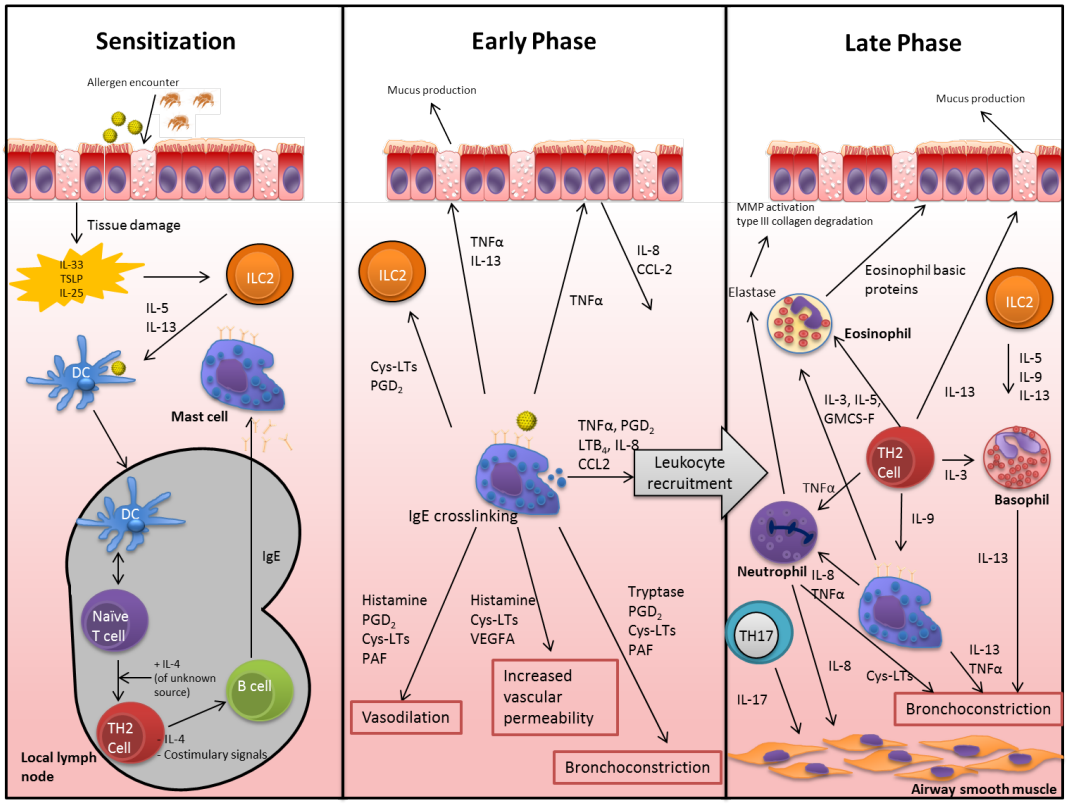

Figure 3.1: Different phases of allergic airway inflammation - The figure is adapted from Galli et al. (2008)

3.3.2.3 Chronic Allergic Inflammation

In case of a repetitive or sustained exposure to an allergen, large numbers of immune cells congregate in the tissue of allergen contact. The following dysregulated, prolonged inflammation can ultimately have diverse effects on the stromal and epithelial cells of the affected tissue (Murdoch and Lloyd, 2010). In many allergic diseases, such

as asthma or atopic dermatitis, in which the affected patient is continuously exposed to an allergen, the prolonged inflammation can result in a process called tissue remodeling. In case of asthma the term tissue remodeling describes a misguided healing process that results in a strong functional impairment of the lung. On a cellular level, this process is based on deep structural changes of the lung tissue. Amongst others, neovascularization, an increase of smooth muscle cells and fibrosis can be observed in patients that suffer from chronic inflammation in the lung (Vignola et al., 2000).

3.4 Treatment Strategies for Allergic Disorders

The treatment strategies for allergic diseases can be classified into two different main approaches. Firstly, different drugs aim at improving the allergic symptoms via the suppression of allergic inflammation. Secondly, allergen-specific immunotherapy (AIT) aims at inducing a sustained allergen-specific tolerance. The following section will highlight the mechanisms of both therapeutic approaches.

3.4.1 Symptomatic and Anti-Inflammatory Treatments

There are several drug classes that are used for the symptomatic treatment of allergic diseases. The most prominent medications are H_1 antihistamines. These molecules act as inverse antagonists of the H_1 receptor and competitively inhibit histamines from binding to the receptor (Baroody and Naclerio, 2000). The suppression of allergic inflammation is achieved via direct or indirect effects on nuclear factor-κB signaling that results in reduced antigen-presentation and decreased secretion of pro-inflammatory cytokines (F. E. Simons and K. J. Simons, 2008). Another symptomatic medication that is used in allergic asthma and allergic rhinitis are the leukotriene receptor antagonists (LTRA). Leukotrienes are lipid mediators derived from the arachidonic acid metabolism and characterized by their pro-inflammatory and bronchioconstrictory effects (Funk, 2001). Leukotriene antagonists which competetively bind to cysteinyl leukotriene 1 (CysLT1) receptor have been proven to be efficient in the treatment of certain allergic conditions of the lung (Scott and Peters-Golden, 2013). The best known drug of this class is Montelukast, which is used in asthma control and allergic rhinitis. Another class of drugs that is frequently used in the treatment of allergic inflammation are glucocorticoids. These lipophilic hormones transmit their anti-inflammatory properties via

different mechanisms. The most prominent mechanism of action is the genomic mode of action: After binding of glucocorticoids to the glucocorticoid receptor, the complex translocates into the nucleus where it builds homodimers and binds to specific promotor regions. As a consequence, different anti-inflammatory cytokines are produced whereas pro-inflammatory cytokines are downregulated (Stahn and Buttgereit, 2008). Besides this pathway, different incompletely understood non-genomic pathways somehow contribute to the glucocorticoid-transmitted immune suppression. These mechanisms include binding of cytosolic glucocorticoid receptors, binding to membrane-bound glucocorticoid receptors, and binding to other membrane-bound proteins or lipids (Boardman et al., 2014). These symptomatic treatments are well-known for their ability to improve the quality of life of affected people. Additionally, a well dosed suppression of inflammatory responses can even prevent the progression of certain allergic diseases (Chetta et al., 2003). However, these symptomatic treatments have the disadvantage that they don´t represent a causal treatment of allergic diseases and have to be taken permanently by patients.

3.4.2 Allergen-Specific Immunotherapy

To this day, allergen-specific immunotherapy represents the only curative treatment of IgE-mediated allergic diseases. This fact is exceptional, as the very same therapy concept that was introduced in 1911 by Leonard Noon (Noon, 1911) still represents the first choice for the treatment of today´s allergic patients. Then and now, allergic patients were treated with repeated subcutaneous injections of increasing allergen extract doses. Even though this therapy can reflect upon a long and successful clinical history, the exact mechanisms of action remain only incompletely understood. Nevertheless, numerous hallmarks of a successful AIT have been successfully identified within the last years: Early desensitization of mast cells and basophils, regulatory T cell induction, expression of allergen-specific IgG antibodies, suppression of eosinophil activation and migration and regulatory B cell generation have all been described as part of the molecular mechanism of AIT (van de Veen, 2017). A better understanding of the decisive markers of a successful therapy is of great clinical interest, as the efficacy of AIT strongly varies in different diseases. An indicative example for these differences is the diverse outcome of AIT for hymenoptera allergy and AIT for house dust mite allergy. Treatment with hymenoptera venom displays a much higher therapeutic efficacy than

AIT with house dust mite extract (Sahin et al., 2017). The following paragraphs will elucidate the role of regulatory B and T cells in the course of AIT.

3.4.2.1 Regulatory T Cells in Allergen-Specific Immunotherapy

The importance of regulatory T cells for the tolerance induction during AIT is well accepted by now. Francis et al., 2003 observed that successful AIT results in the induction of IL-$10^+$$CD4^+$$CD25^+$ T cells via grass pollen immunotherapy. Further on, (Jutel, M. Akdis, et al., 2003) could show that allergen-specific $FoxP3^+$ regulatory T cells are crucially involved in a successful AIT. Different studies could demonstrate that regulatory T cells are capable of diminishing allergic TH2 responses (C. A. Akdis et al., 1998). The suppressive effect of these T cells could be linked, amongst others, to their their secretion of anti-inflammatory cytokines, such as Interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). These well characterized cytokines are known to have numerous suppressive effects on different cells of the innate and adaptive immune system.

IL-10 is not only able to suppress IgE production (Punnonen et al., 1993) by B cells and pro-inflammatory cytokine expression by T cells (Schandene et al., 1994), but is also involved in the downregulation of costimulatory molecules and major histocompatibility complex II (MHC-II) on APC´s (de Waal Malefyt et al., 1991), blocking of co-stimulatory signals on T cells (Taylor et al., 2007) and suppression of mast cells, eosinophils and basophils (Larche et al., 2006).

TGF-β family members on the other hand, are well known for their importance in the expansion and regulation of $CD4^+$ $CD25^+$ cells (Fu et al., 2004). It was shown that TGF-β1 reduces FcϵRI expression on mast cells (Gomez et al., 2005). Furthermore, Coffman et al. (1989) discovered that TGFβ1 specifically enhances IgA production while suppressing IgE secretion.

Besides their expression of anti inflammatory cytokines, regulatory T cells are able to directly suppress mast cell degranulation via OX40-OX40L interaction (Gri et al., 2008). All these observations, which only represent a fraction of described regulatory mechanisms of regulatory T cells, emphasize the outstanding role of regulatory T cells in the course of tolerance induction during AIT and make the induction of regulatory T cells an attractive therapeutic aim.

3.4.2.2 Regulatory B Cells and the Humoral Immune Response in Allergen Specific Immunotherapy

More recent studies have identified regulatory B cells (Bregs) as an additional regulatory cell type that is strongly involved in AIT-mediated tolerance induction (Boonpiyathad et al., 2017). Similar to regulatory T cells, Bregs act through the secretion of anti-inflammatory cytokines like IL-10 and TGF-β. Additionally, Bregs secrete IL-35, an anti-inflammatory cytokine that induces a strong regulatory T cell response (Collison et al., 2010).

Besides the secretion of anti-inflammatory cytokines, Bregs are able to suppress allergic symptoms by the production of allergen-specific high affinity IgG antibodies. Studies performed by (R. A. Cooke et al., 1935) showed that serum from AIT-treated patients contain allergen-specific factors that suppress an immediate type inflammation of the skin. A few years later, studies revealed that this protective effect is mediated by allergen-specific antibodies of the IgG isotype (Lichtenstein et al., 1968). By now, it is well established that a rise of IgG4 immunoglobulins during allergen-specific immunotherapy is associated with a decrease of allergic symptoms (Jacobsen et al., 2012). The most widespread explanation for this observation is the blocking capacity of this antibody subtype (Aalberse et al., 2009). This blocking capacity of IgG4 is, amongst others, based on its ability to bind and neutralize the allergen before IgE binding (Flicker and Valenta, 2003). Further on, its anti-inflammatory capacity is due to a mechanism called "Fab arm exchange". In this process, in which IgG4 antibodies exchange half-molecules, immunoglobulins with two different variable regions arise (Rispens et al., 2011). These bi-specific antibodies prevent allergen-mediated cross-linking of mast cell receptors. Additionally, it could be shown that IgG4 is able to prevent IgE-facilitated allergen presentation that results in an increased T cell activation (van Neerven et al., 1999). Besides the blocking effect, it is known that distinct immunoglobulin subtypes, e.g. IgG4, suppress mast cell degranulation by binding to the inhibitory Fcγ receptor IIB (FcγRIIB) (Takai et al., 2003). A very recent study revealed that the secretion of these blocking antibodies of the IgG4 isotype in humans is restricted to a IL-10^+ regulatory B cell population (van de Veen et al., 2013).

To this day, these so called blocking antibodies and their ratio to allergen-specific IgE antibodies were targeted by numerous studies (James et al., 2011). However, due to indistinct results of these studies, the importance of the ratio of allergen specific

blocking antibodies to IgE antibodies for an effective AIT remains unclear (Shamji et al., 2017). Nevertheless, the involvement of regulatory B cells and the rise of IgG4 has become a distinct feature in the course of effective AIT.

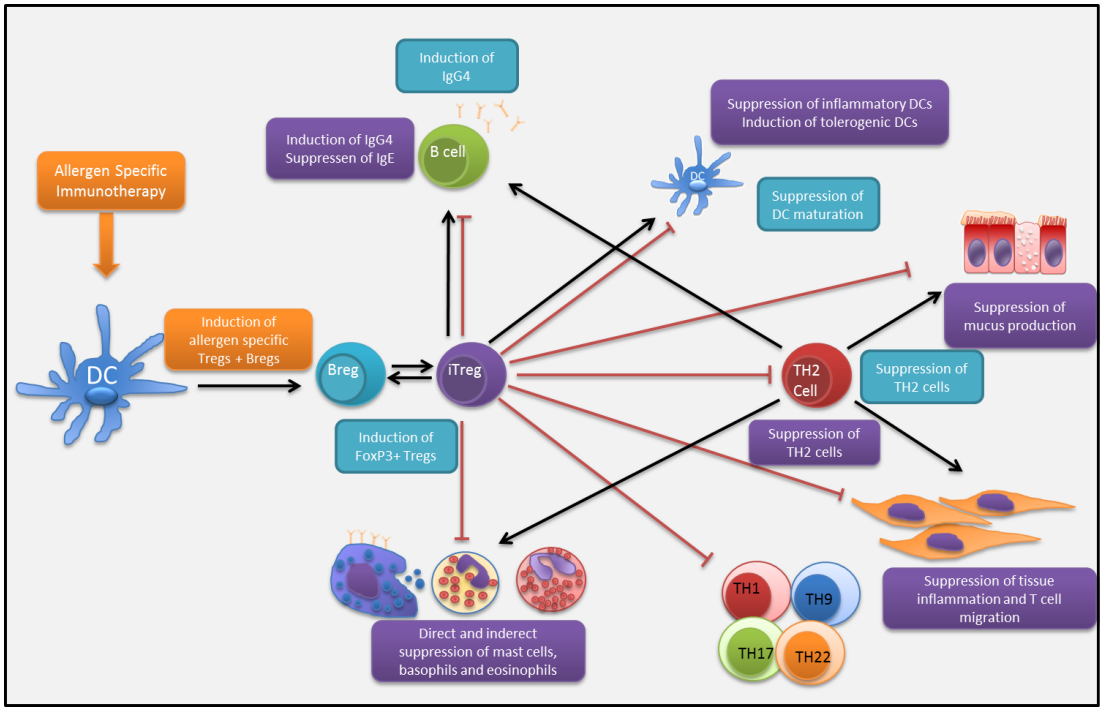

Figure 3.2: Mechanisms of AIT - The figure is adapted from M. Akdis and C. A. Akdis, 2014.

3.4.3 Needs in Allergen-Specific Immunotherapy

Even though AIT represents the only causal treatment for allergic diseases, it is characterized by several shortcomings that have not been overcome, yet. Firstly, AIT goes along with a risk of anaphylaxis (Bernstein et al., 2004). Further, AIT is not efficient in all patients and for all allergies. The heterogeneous pathologies underlying different allergic diseases in different patients are not all treatable with the same AIT. Additionally to the safety risk and the varying treatment efficiency, the long treatment time can result in low patient compliance. Different novel approaches aim at solving these AIT-related problems regarding safety, efficiency and treatment time (Jutel, Kosowska, et al., 2016). The approach of this dissertation examines the effect of the combined

administration of AIT with a IL-4 receptor antagonist (IL-4 Mutein) on tolerance induction.

3.5 The IL-4/IL-13 Receptor Complex

The IL-4/IL-13 receptor complex has been intensely studied within the last years. One reason for the extensive interest in this receptor complex is the fact, that it is crucial for the signaling of the two prominent TH2 cytokines IL-4 and IL-13. On a molecular level, the relevance of this receptor complex for signaling of two cytokines can be explained via a receptor subunit shared by IL-4 and IL-13. The IL-4-receptor-α (IL-4R-α) chain can bind IL-4 and subsequently dimerize with either the common-γ chain (γ_c) or with the IL-13 receptor-α-1 (IL-13Rα-1) subunit, forming the type I or type II receptor complex for IL-4. Furthermore, the IL-4-reptor-α subunit can bind to a dimer of IL-13 and a secreted form of the IL-13 receptor-α-1 chain to form another type II complex. Additionally to these functional receptors, different decoy receptors for IL-4 and IL-13 could be identified. Mosley et al., 1989 could show that soluble IL-4R-α chain binds IL-4 and suppresses IL-4-mediated signaling. The IL-13-receptor-α-2 chain (IL-13R-α-2) which is lacking intracellular signaling subunits on the other hand, acts as decoy receptor for IL-13 and suppresses IL-13-mediated signaling (Yoshikawa et al., 2003).

The intracellular signaling pathways of the differing receptor types are slightly different. Whereas dimerization of the type I receptor results in the activation of receptor associated Janus Kinases of the types JAK1, JAK2 and JAK3, the type 2 receptor complex is characterized by the activation of JAK1, JAK2 and Tyk3 (Heller, 2012). Additionally, different members of the Signal Transducer and Activator of Transcription (STAT) family can be activated by IL-4 and IL-13 binding. Most predominant is the activation of STAT6. Mice deficient for STAT6, which was formerly known as IL-4-STAT, are unable to generate TH2 cells or to initiate an effective IgE response (Kaplan, Whitfield, et al., 1998). Additionally, activation of other members of the STAT family, such as STAT1a (Wang et al., 2004), STAT3 (Orchansky et al., 1999) and STAT5 (Roy et al., 2002) are associated with IL-4 receptor signaling.

Even though IL-4 and IL-13 are structurally very similar and share the same receptor complex, they have different, only partially redundant, functions. As mentioned in chapter 1.3.2, IL-4 is widely known as the TH2 key cytokine *per se*. When Mosmann

and his colleagues (Mosmann et al., 1986) described and denominated TH2 cells for the very first time, IL-4 - formerly known as B cell stimulting factor1 (BCSF1) - was identified as TH2-specific cytokine. Today, after many years of intensive research, much more is known about IL-4 and its biological functions. Amongst others, it could be shown that IL-4 is not only crucial for the class switch to IgE production in B cells (Armitage et al., 1992), but also for the differentiation of naive $CD4^+$ T cells to TH2 cells (Kaplan, Schindler, et al., 1996). Furthermore, numerous *in vitro* studies could show, that IL-4 suppresses the expression of the Treg transcription factor FoxP3 (Dardalhon et al., 2008), (Mantel et al., 2007), which is crucial for the development of Tregs.

IL-13 was discovered shortly after IL-4 (Cherwinski et al., 1987). In allergic inflammation, IL-13 is present in much higher concentrations than IL-4 (Munitz et al., 2008). Nevertheless, it took more then 10 years to demonstrate its unique importance in allergic lung inflammation. The group around Jack Elias could proof that IL-13 is not only crucial for mucus hyperproduction, but also for the majority of asthma symptoms (Zhu et al., 1999).

There are several hypotheses that aim at explaining the different physiological effects of the two cytokines which share the type II receptor. One theory suggests that the different biological functions of IL-4 and IL-13 are caused by different affinities of the cytokines to the IL-4 receptor-α chain and the IL-13 receptorα1 chain. Additionally, it is thought that the differences are caused by varying relative abundances of type I and type II receptor complexes on different cell types (Heller, 2012). More recent studies could prove that IL-4 and IL-13 are actually able to induce different intracellular signaling pathways via binding to the same type II receptor heterodimer (LaPorte et al., 2008). A more complete understanding of the unique roles of IL-4 and IL-13 signaling is particularly interesting for the development of novel precise therapeutic approaches for atopic diseases.

3.6 The IL-4/IL-13 Receptor Complex as Therapeutic Target

The importance of IL-4 and IL-13 receptor signaling in human atopic diseases could primarily be shown in a well-designed study that associated a gain of function mutation of the IL-4 receptor with atopy (Hershey et al., 1997). By now, it is known that

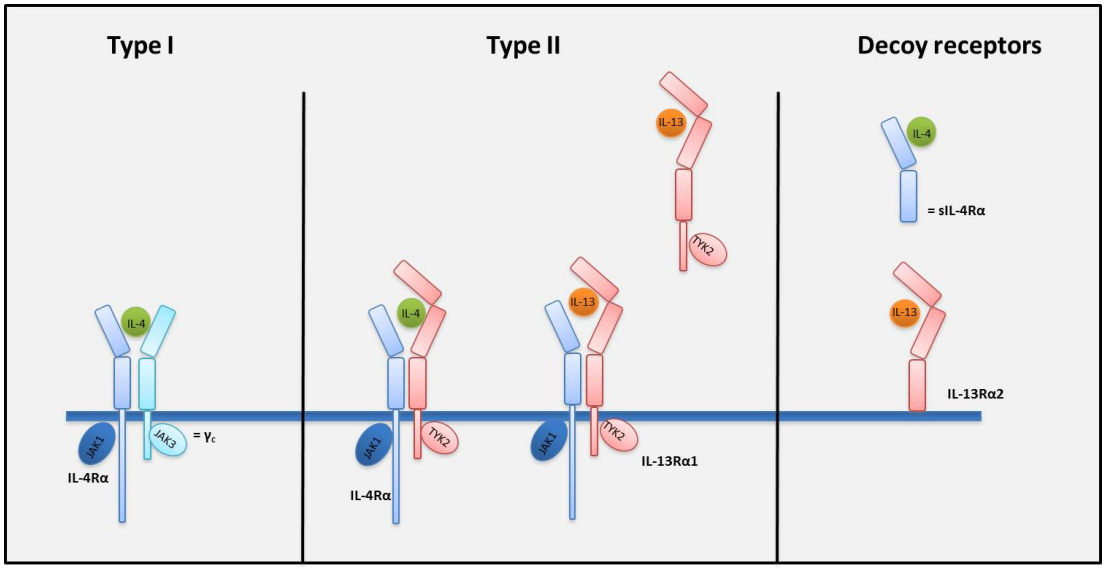

Figure 3.3: The IL-4/IL-13 receptor complex - The figure is adapted from Heller, 2012

IL-4/IL-13 receptor signaling is involved in numerous processes that are decisive for allergic diseases: As mentioned in chapter 1.3, the receptor complex is indispensable for TH2 differentiation, the IgE switch of B cells and the majority of symptoms of allergic inflammation. This miscellaneous panel of biologic functions of the IL-4/IL-13 receptor complex makes it a very attractive therapeutic target in atopic diseases. The first approaches with biologicals targeted either IL-4 or IL-13 signaling. Amongst others, a monoclonal antibody against IL-4 (SB 240683) was tested in a phase 1 study with asthmatic patients but did not meet the expectations (Steinke, 2004). Several IL-13 specific monoclonal antibodies (anrukinzumab, lebrikizumab, tralokinumab) are at different stages of clinical studies at this time. The preliminary results are varying regarding the improvement of asthma symptoms (Bagnasco et al., 2016). Additionally, different approaches to block the signaling of both, IL-4 and IL-13, were initiated. A soluble form of IL-4 receptor-α was shown to suppress IL-4-mediated signaling while slightly enhancing IL-13 signaling as it stabilizes suboptimal doses of IL-13 (Andrews et al., 2006).

Finally, a monoclonal antibody that targets IL-4 and IL-13 signaling proved to be highly efficient: Dupilumab, a fully human monoclonal antibody binds to the IL-4 receptor-α chain which is indispensable for IL-4 and IL-13 signaling. Several clinical

studies resulted in striking improvements in different atopic diseases: In atopic dermatitis, therapy with dupilumab resulted in rapid, significant improvements in all observed parameters (Beck et al., 2014). More recently, a study with patients suffering from severe persistent asthma showed that Dupilumab treatment induces an increased lung function and reduces severe exacerbations (Wenzel, Castro, et al., 2016). It is well imaginable that Dupilumab, which is already commercially available, could be effective in further atopic diseases.

3.6.1 IL-4 Mutein

Another biological that targets IL-4 and IL-13 signaling at the same time is IL-4 Mutein (Pitrakinra). This Interleukin-4 variant with two point mutations was firstly developed for human applications by Duschl, 1995. In the human variant, the amino acids arginine 121 and tyrosine 124 are substituted by aspartic acid residues. The resulting cytokine variant binds to the IL-4 receptor-α chain but prevents receptor dimerization with the common-γ chain or the IL-13-receptor-α-1 chain and therefore prevents the signal transduction of both cytokines. An analogue for the murine system was developed in 1997 by Grunewald and colleagues (Grunewald, Kunzmann, et al., 1997). The murine variant displays the same biological function as its human counterpart. The murine Mutein is characterized by the replacement of the amino acids glutamine 116 and tyrosine 119 with aspartic acid residues.

Numerous animal studies resulted in promising observations and indicate that IL-4 Mutein could be an interesting biological for the treatment of diverse atopic diseases (Grunewald, Werthmann, et al., 1998) (Hahn et al., 2003), (Tomkinson et al., 2010). Additionally, two clinical phase 2a studies focusing on the subcutaneous and inhalative treatment with IL-4 Mutein resulted in slight improvements of patients suffering from atopic asthma (Wenzel, Wilbraham, et al., 2007). In contrast to most of these studies, which focused solely on the improvement of symptoms of allergic inflammation, the approach of this thesis aims at sustainably modulating the allergic immune response. Via combination of AIT with a prolonged administration of IL-4 Mutein this project intends to skew the allergen-specific T cell response from a TH2 phenotype to a regulatory phenotype.

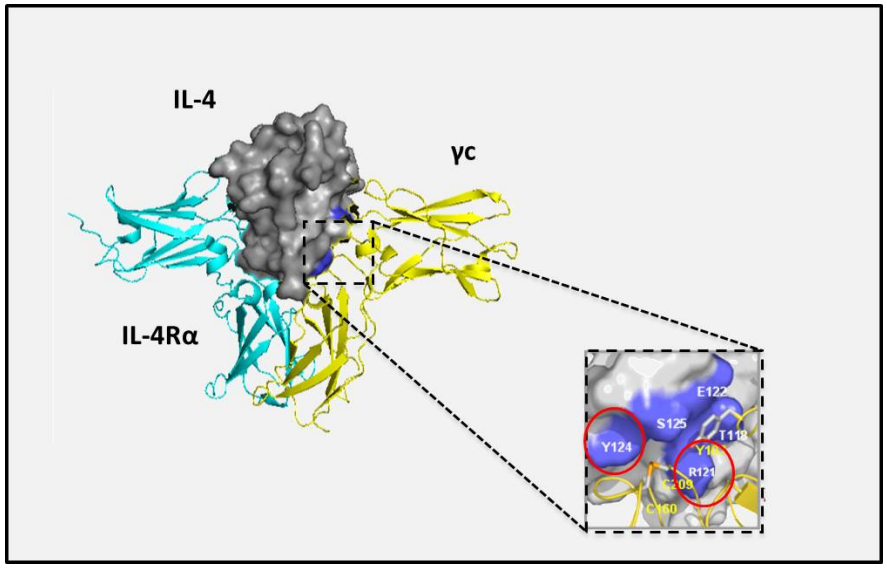

Figure 3.4: Formation of the type I receptor complex - Enlarged are the amino acids that are substituted in the human IL-4 Mutein. The figure is adapted from LaPorte et al., 2008

3.7 Thermosensitive PLGA-PEG-PLGA Triblock Copolymer Based Hydrogel as Delivery Matrix for Allergens and Biologicals during AIT

Aluminum hydroxide (alum) is widely used as depot-adjuvants for vaccines and allergen-matrix during AIT (Kramer and Heath, 2014). However, within the last years several drawbacks of the high alum exposure during long term AIT aroused the attention of scientists. Numerous unwanted effects of a high aluminium uptake on the organism have been described (Krewski et al., 2007). It is not only for this reason, that this project aimed at evaluating a novel alum-free, depot-forming delivery matrix for AIT. Additionally, a depot-forming delivery matrix is decisive for the sustained administration of biologicals with short serum half-lifes such as IL-4 Mutein. The chosen delivery matrix in this project is a thermosensitive PLGA-PEG-PLGA triblock copolymer-based hydrogel that has been well described by (Jeong et al., 2000). This polymer forms a non-immunogenic, biodegradable and thermosensitive hydrogel in water and could be shown to be a suitable delivery matrix for interleukin-2 *in vivo* (Qiao, D. Chen, Hao, et al., 2008). Aim of this study was the synthesis and characterization of the PLGA-PEG-

PLGA polymer. Furthermore, a PLGA-PEG-PLGA based hydrogel should be tested for its suitability as delivery matrix for the model allergen OVA and the biological IL-4 Mutein during a murine model of AIT.

4

Aims of the project

4.1 Final Aim

The ultimate goal of this dissertation is the combination of sustained IL-4 receptor signal-inhibition during AIT in a model of allergic lung inflammation with increased serum levels of IL-4 and IL-13. Further, the potential therapeutic benefit of IL-4 Mutein administration during AIT should be analyzed in detail.

4.2 Preliminary Aims

- Recombinant expression of IL-4 Mutein in a eukaryotic expression system
- Analysis of the biological activity of IL-4 Mutein *in vitro* and *in vivo*
- Synthesis of a PLGA-PEG-PLGA triblock copolymer based thermosensitive hydrogel
- Evaluation of the PLGA-PEG-PLGA triblock copolymer based hydrogel as depot-forming protein delivery matrix *in vitro* and *in vivo*
- Examination of the effect of IL-4 Mutein administration on a traditional model of AIT
- Establishment of a model of AIT during chronic allergen exposure
- Analysis of the therapeutic outcome of AIT in a chronic model of lung inflammation in presence and absence of IL-4 Mutein

5

Methods

5.1 Animal Procedures

5.1.1 Laboratory Animals

Female C57BL/6 mice between 5-7 weeks of age were purchased from Charles River (Sulzfeld, Germany). Animals were kept in individually ventilated cages (VentiRack, Biozone, Margate/UK) under specific pathogen free (SPF) conditions. At the start of the experiments, mice were aged between 6-8 weeks. All experiments were carried out under federal guidelines for the use and care of laboratory animals and approved by the government of the district of upper Bavaria (Reference numbers of ethical approvals for all performed animal experiments are 55.2-1-54-2532-50-2017 and 55.2-1-54-2532-104-13. The majority of *in vivo* experiments was performed in room 6116, E-Streifen (KTH), Zentrale Versuchstierhaltung des Helmholtz Zntrum München, Ingolstädter Landstraße 1, 85764 Neuherberg. The experiments "IL-4 Mutein prevents Alum-mediated sensitization to Ovalbumin *in vivo*" (results section 7.3) and "Effect of IL-4 Mutein on a murine model of allergen-specific immunotherapy" (results section 7.5) were performed in the facilities of the German Mouse Clinic,Ingolstädter Landstrasse 1, 85764 Neuherberg.

5.1.2 Animal Handling

Intraperitonel injection

I.p. injection was performed in the lower right quadrant of the abdomen. A maximum volume of 200 µl was injected per treatment.

Subcutaneous injection

Subcutaneous administration of material was carried out into the loose skin over the neck between the shoulders.

Intranasal instillation

Mice were anesthetized with Sevoflurane. Subsequently 10 µl of solution was slowly administered to each nostril using a 10 µl pipette.

Anesthesia

Mice were anesthetized by i.p. injection of 10% Ketamine/2% Xylacin in phosphate buffered saline (PBS). 100 µl of preparation was injected per 10 g body weight. For short term anesthesia Sevoflurane inhalation was applied.

Euthanasia

The preferred method for euthanasia of mice was cervival dislocation. If mice had to undergo BAL analysis, an overdose of injectable anesthetic was used leading to a death without regaining consciousness.

Blood collection

Blood was collected via puncture of the orbital sinus or plexus. Not more than 15% of the calculated blood volume was collected from Sevoflurane anesthetized mice. Blood was collected in Microvette® serum tubes (Sarstedt, Nümbrecht, Germany), centrifuged at 7000 *G* and stored at -80 ℃.

Exposure to nebulized allergen in a challenge chamber

Not more then 10 mice at the same time were transferred to a 22 cm x 21 cm x 12,5 cm sized acrylic glass chamber and exposed to a 1% ovalbumin (OVA) aerosol that was nebulized using a Pariboy® (PARI Respiratory equipment, Midlothian, TX, USA) for 15 min.

5.1.3 Animal Models of Allergic Diseases

5.1.3.1 Effect of IL-4 Mutein on the Alum Mediated Sensitization to the Model Allergen Ovalbumin

In order to induce an OVA-specific TH2 immune response in the positive control group, mice were intraperitoneally (i.p.) injected with 200 µl of 10 µg OVA complexed with

2 mg aluminum hydroxide (alum) in PBS. Mice in the negative control group were i.p. injected with PBS/alum. Mice in the test group were treated with 100 µg IL-4 Mutein i.p. injections 2 h before and 2 h after the sensitization. 100 µg IL-4 Mutein was further i.p. administered on days 2, 4, 6 and 8. Mice from all groups were euthanized by an overdose of injectable anesthetic and cervical dislocation on day 10.

5.1.3.2 Effect of IL-4 Mutein on Allergen Challenge in a Model of House Dust Mite Induced Allergic Airway Inflammation

Mice in the positive control group were sensitized on day 1 by intranasal (i.n.) administration of 1 µg house dust mite (HDM) extract. Mice were challenged on days 7, 8, 9, 10 and 11 with 10 µg HDM extract (i.n.). Mice from the IL-4 Mutein therapy group were sensitized on day 1 with 1 µg HDM extract and challenged on days 7, 8, 9, 10 and 11 with 10 µg HDM extract. Additionally, these mice received 100 µg of IL-4 Mutein (i.p.) 1 h prior to each allergen challenge. Mice were euthanized for analysis on day 15.

5.1.3.3 Murine Model of Allergen-Specific Immunotherapy

Mice from the negative control group were sensitized with 2 mg alum and PBS on days 1 and 7 (i.p.). Subsequently, mice received two injections of PBS on days 21 and 28 (s.c.). Mice from the positive control group were sensitized with OVA/alum (i.p.) and received two injections of PBS on days 21 and 28 (s.c.). Mice from the OVA-therapy group were sensitized with OVA/alum (i.p.). Two injections of 500 µg OVA (s.c.) in the thermosensitive hydrogel served as allergen specific immunotherapy (AIT) in this group. Mice that received AIT in combination with IL-4 Mutein received two injections of 500 µg OVA and 100 µg IL-4 Mutein (both dissolved in a 20% PLGA-PEG-PLGA polymer solution) on days 21 and 28 (s.c.). On days 42, 45 and 48 mice were challenged for 15 min with a nebulized 1% OVA in PBS solution. Euthanasia and read-out followed on day 49.

5.1.3.4 Murine Model "Therapeutic Targets of IL-4 Mutein"

Mice from the "Unchallenged" group were sensitized with OVA/alum on days 1, 7 and 21 (i.p.). Mice from this group were challenged on days 42, 45 and 48 for 15 min with PBS aerosol. Animals from the "Challenged" group were sensitized with OVA/alum on

days 1, 7 and 21 (i.p.). Subsequently, they were challenged for 15 min with a nebulized 1% OVA solution on day 48. All mice were euthanized on day 49.

5.1.3.5 Murine Model "Short Term Effect of IL-4 Mutein during AIT and Repetitive Allergen Exposure"

Mice from the negative control group were sensitized on days 1, 7 and 21 with PBS/alum (i.p.). Subsequently, they received 200 µl injections of PBS on days 35, 38 and 41 (s.c.). Mice from the positive control group were sensitized on days 1, 7 and 21 with OVA/alum (i.p.). Subsequently, they received 200 µl of PBS on days 35, 38 and 41 (s.c.). Mice from the OVA-therapy group were sensitized on days 1, 7 and 21 with OVA/alum (i.p.). Subsequently, they received 500 µg OVA in 200 µl PBS on days 35, 38 and 41 (s.c.). Mice from the OVA-therapy + IL-4 Mutein group were sensitized on days 1, 7 and 21 with OVA/alum (i.p.). Subsequently, they received subcutaneous injections of 500 µg OVA in 200 µl PBS on days 35, 38 and 41 (s.c.). On days 34-42 these mice received daily i.p. injections of 100 µg IL-4 Mutein in 100 µl PBS.

On days 34, 37 and 40 mice of all groups were challenged for 15 min in a challenge chamber with 1% nebulized OVA. Read-out was performed on day 43.

5.1.3.6 Murine Model "Long Term Effect of IL-4 Mutein during AIT and Repetitive Allergen Exposure"

Mice from the negative control group were sensitized on days 1, 7 and 21 with PBS/alum (i.p.). Subsequently they received 200 µl injections of PBS on days 35, 38 and 41 (s.c.). Mice from the positive control group were sensitized on days 1, 7 and 21 with OVA/alum (i.p.). Subsequently they received 200 µl of PBS on days 35, 38 and 41 (s.c.). Mice from the OVA-therapy group were sensitized on days 1, 7 and 21 with OVA/alum (i.p.). Subsequently they received 500 µg OVA in 200 µl PBS on days 35, 38 and 41 (s.c.). Mice from the OVA-therapy + IL-4 Mutein group were sensitized on days 1, 7 and 21 with OVA/alum (i.p.). Subsequently they received subcutaneous injections of 500 µg OVA in 200 µl PBS on days 35, 38 and 41 (s.c.). On days 34-42 these mice received daily i.p. injections of 100 µg IL-4 Mutein in 100 µl PBS.

On days 34, 37 and 40 mice of all groups were challenged for 15 min in a challenge chamber with 1% nebulized OVA. Additionally, a second challenge block with 1% neb-

ulized OVA was performed on days 56, 59 and 62. Mice were euthanized for analysis on day 63.

Group	Sensitization	Treatment
Negative Control	PBS/2 mg alum	PBS
Positive Control	10 µg OVA/2 mg alum	PBS
IL-4 Mutein Therapy	10 µg OVA/2 mg alum	100 µg IL-4 Mutein

Table 5.1: Treatment groups in the murine model "Prophylactic IL-4 Mutein treatment during OVA/alum sensitization"

Group	Sensitization	Challenge	Treatment
Positive Control	1 µg HDM extract	10 µg HDM extract	PBS
IL-4 Mutein Therapy	1 µg HDM extract	10 µg HDM extract	100 µg IL-4 Mutein

Table 5.2: Treatment groups in the murine model "Effect of IL-4 Mutein treatment on house dust mite induced lung inflammation"

Group	Sensitization	Treatment	Challenge
Negative Control	PBS/alum	PBS	1% OVA aerosol
Positive Control	OVA/alum	PBS	1% OVA aerosol
OVA-Therapy	OVA/alum	500 µg OVA	1% OVA aerosol
OVA-Therapy + IL-4 Mutein	OVA/alum	500 µg OVA + 100 µg IL-4 Mutein	1% OVA aerosol

Table 5.3: Treatment groups in the murine model "Allergen-specific immunotherapy"

Group	Sensitization	Challenge
Unchallenged	OVA/alum	PBS
Challenged	OVA/alum	1% OVA aerosol

Table 5.4: Treatment groups in the murine model "Therapeutic targets of IL-4 Mutein"

Group	Sensitization	Treatment	Challenge
Negative Control	PBS/alum	PBS + PBS	1% OVA aerosol
Positive Control	OVA/alum	PBS + PBS	1% OVA aerosol
OVA-Therapy	OVA/alum	500 µg OVA + PBS	1% OVA aerosol
OVA-Therapy+ IL-4 Mutein	OVA/alum	500 µg OVA + 100 µg IL-4 Mutein	1% OVA aerosol

Table 5.5: Treatment groups in the murine model "Short term effect of IL-4 Mutein and AIT on allergic lung inflammation after repetitive allergen exposure"

Group	Sensitization	Treatment	Challenge
Negative Control	PBS/2 mg alum	PBS + PBS	1% OVA aerosol
Positive Control	30 µg OVA/ 2 mg alum	PBS + PBS	1% OVA aerosol
OVA-Therapy	OVA/alum	500 µg OVA + PBS	1% OVA aerosol
IL-4 Mutein Therapy	OVA/alum	PBS + 100 µg IL-4 Mutein	1% OVA aerosol
OVA-Therapy+ IL-4 Mutein	OVA/alum	500 µg OVA + 100 µg IL-4 Mutein	1% OVA aerosol

Table 5.6: Treatment groups in the murine model "Long term effect of IL-4 Mutein and AIT on allergic lung inflammation after repetitive allergen exposure"

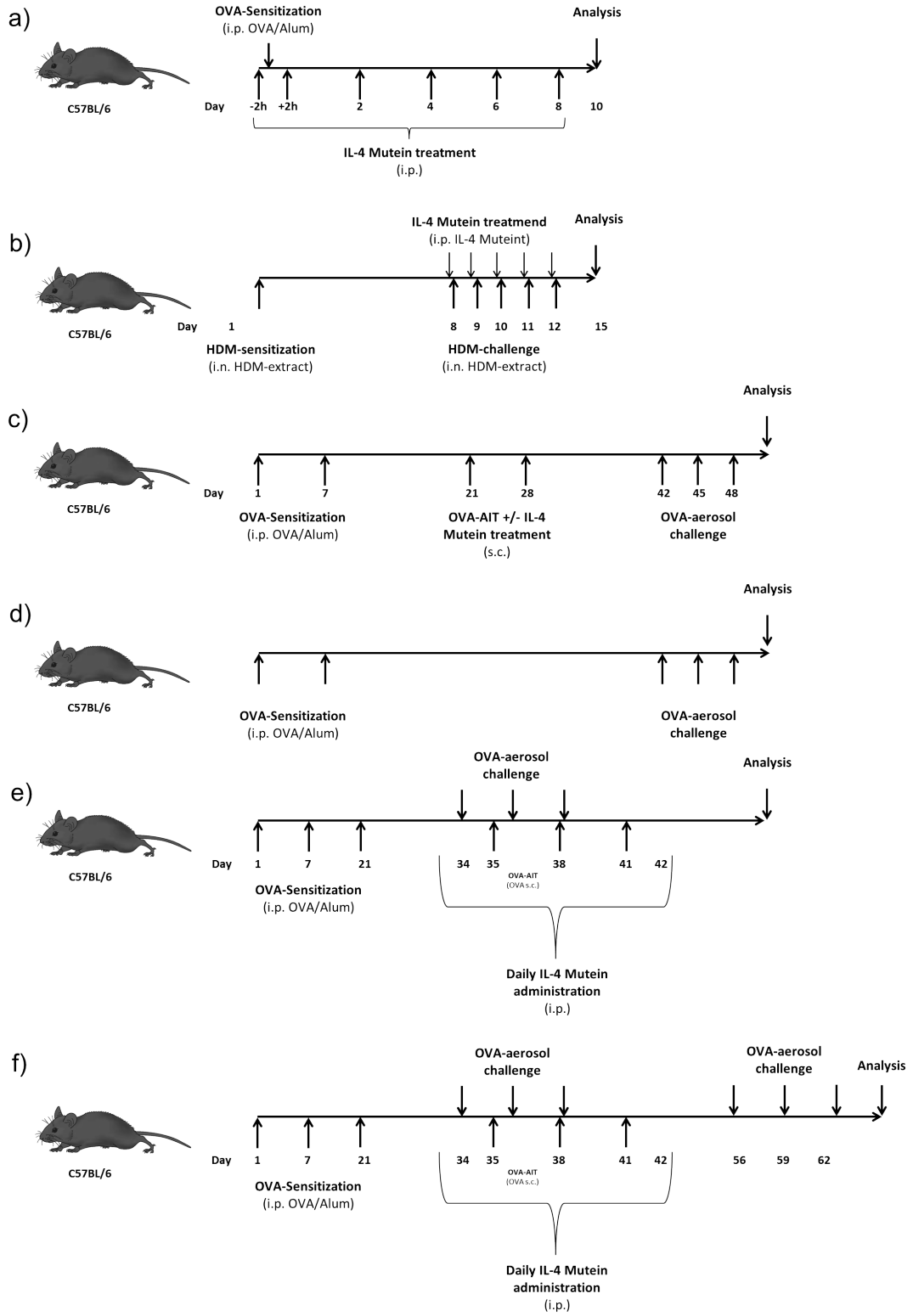

Figure 5.1: Overview murine models - Detailed descriptions regarding n-number and treatment groups can be found in the corresponding results chapters

5.2 Cell Biology Methods

5.2.1 Isolation of Leukocytes from Lung Tissue for Flow Cytometric Analysis

Lungs were removed from mice and placed in a six-well plate. A few drops of Roswell Park Memorial Institute medium (RPMI) were added and lungs were chopped into approximately 1 mm x 1 mm sized pieces. 3 ml digestion medium was added to the lungs and incubated at 37 °C under gentle shaking. Digestion was stopped by the addition of 15 µl 0,5 M EDTA. Digested lungs were transferred to a cell strainer and mashed with a syringe piston into a 50 ml tube. Cell strainer was washed with 3 ml RPMI. Next, cells were pelleted (400 *G*, 4 °C, 5 min) and resuspended in 5 ml 40% percoll in RPMI (v/v) solution. A long glass pasteur pipette was placed in the 15 ml tube and the 40% percoll solution was underlayerd with 5 ml 80% percoll solution. Tubes were centrifuged (1600 *G*, RT, 15 min) with brake set to 1. Lymphocytes were collected from the interphase and washed with PBS.

5.2.2 Isolation of Leukocytes from Lymph Nodes

Lymph nodes were removed from mice and placed in 5 ml RPMI. Next, lymph nodes were mashed with a syringe piston through a cell strainer (70 µm). Thereafter, cell strainer was washed with 5 ml RPMI. For further use, cells were washed twice with PBS.

5.2.3 Isolation of Leukocytes from Spleen

Spleens were removed from mice and placed in 5 ml RPMI. Spleens were mashed with a syringe piston through a cell strainer (70 µm). Thereafter, cell strainer was washed with 10 ml RPMI. In order to remove erythrocytes, cells were pelletted and resuspended in 0,5 ml ACK-lysis buffer. After a 30 s incubation time at RT, 10 ml PBS was added. For further use, cells were washed twice with PBS.

5.2.3.1 *In vitro* TH2 Differentiation

Flat bottom 96 well plates were coated with anti-CD3 and anti-CD28 antibodies overnight at 4 ℃. Naive T cells from 4get IL-4 reporter mice were isolated from splenocytes according to the MACS manual of the naive T cell $CD4^+$ isolation kit (Naive $CD4^+$ T

Cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). Living cells were counted using trypan blue staining in a haemacytometer. 200.000 cells were plated out per well. TH2 polarization of naive $CD4^+$ T cells was achieved by the addition of 10 ng/ml IL-4 and 3.4 µg/ml anti IFNγ. Cells were cultured at 37 ℃, 5% CO_2 and a relative humidity of 95% for three days. On day three 100 µl of medium were replaced with 100 µl IL-4 and anti IFNγ containing medium. Cells were cultured for two more days and subsequently analyzed via FACS.

5.2.3.2 *In vitro* Treg differentiation

Flat bottom 96 well plates were coated with anti-CD3 and anti-CD28 antibodies overnight at 4 ℃. Naive T cells from FoxP3-mCherry reporter mice were isolated from splenocytes according to the MACS manual of the naive T cell $CD4^+$ isolation kit (Naive $CD4^+$ T Cell Isolation Kit, Miltenyi Biotec). Living cells were counted using trypan blue staining in a haemacytometer. 200.000 cells were plated out per well. Treg polarization of naive $CD4^+$ T cells was achieved by the addition of 80 ng/ml IL-2 and 2.4 ng/ml TGF-β and 3.4 µg/ml anti IFNγ. Cells were cultured at 37 ℃, 5% CO_2 and a relative humidity of 95% for three days. On day three 100 µl of medium were replaced with 100 µl IL-4 and anti IFNγ containing medium. Cells were cultured for two more days and subsequently analysed via FACS.

5.2.3.3 CTLL2 Assay

CTLL-2 cells (ATCC® TIB-214™) were cultured in the presence of IL-2 (20 ng/ml). Prior to their application in the IL-4 Mutein functionality assay cells were washed twice with PBS and counted using a haemacytometer. 20.000 cells were plated out per well and stimulated with 5 ng/ml IL-4 and different IL-4 Mutein concentrations. After 48 h of incubation, the amount of living cells was determined according to the manual of the CellTiter-Blue® Cell Viability Assay (Promega, Madison, Wisconsin, USA). More precisely, the amount of metabolically active cells was analyzed using an ELISA plate reader. Fluorescence intensity 560/590 nm was measured and plotted against log(IL-4 Mutein concentration). The IC_{50} value was estimated using GraphPad Prism (four parameter logistic function: log(inhibitor) vs. response - variable slope).

5.2.3.4 Restimulation of Primary Lymphocytes from Lymph Nodes and Spleen

96-well plates were coated with anti-CD3 and anti-CD28 and incubated at 4 °C over night. Plates were washed twice with PBS. Thereafter, lymphocytes from spleens or lymph nodes were plated out at a densitiy of $2x10^5$ cells per well in 200 µl RPMI. Cells were cultured for 72 h at 37 °C, 5% CO_2 in a humidified atmosphere. After 72 h supernatants were collected and cytokine concentrations were quantified by ELISA. Alternatively, $2x10^5$cells per well in 200 µl RPMI were stimulated with different doses of OVA in absence of CD3 and CD28-specific antibodies.

5.2.4 Analysis of Bronchoalveolar Lavage

Chest of euthanized mice was opened. Trachea was cut free and cannulated. Airways were lavaged five times with 0,8 ml PBS. The thorax of the mice was massaged and the PBS was harvested with a syringe. The cell free BAL-fluid of the first aspiration was used for the analysis of cytokines. Cells of all aspirations were pooled, pelleted and washed with PBS for further use.

5.3 Molecular Biology and Protein Chemistry

5.3.1 Recombinant Expression of IL-4 Mutein

Different expression systems were analyzed for their suitability to express IL-4 Mutein. Expression in *Escherichia coli E. coli* (BL21) transformed with the vector Pet22b+resulted in a cytokine inhibitor with very low biological activity. Protein expression in Hek293 cells stably transfected with pcDNA3.1 did not result in an adequate protein yield for *in vivo* experiments. Ultimately IL-4 Mutein was expressed in Dihydrofolate Reductase Deficient ($DHFR^{-/-}$) Chinese Hamster Ovary (CHO) cells.

5.3.1.1 Cloning of Expression Vectors

The coding sequence of IL-4 Mutein with the serum albumin signal peptide and a his-tag was designed using Geneious8.1.5 (Kearse et al., 2012). The construct was ordered with NotI and BamHI overhangs at GeneArt® Strings™(Thermo Fisher, Waltham, Massachusetts, USA). Upon arrival, construct was reconstituted with DNAse free water

to a concentration of 20 ng/µl. Expression vector pIRES-DHFR (vector system in which the gene of interest is coupled via an Internal Ribosomal Entry Site (pIRES) to the selection marker DHFR) and the IL-4 Mutein fragment were digested in a total reaction volume of 20 µl containing FastDigest Buffer green (Thermo Scientific), DNAse free water and restriction enzymes BamHI and NotI. In order to prevent recirculation of digested vector, alkine phosphatase was added to the vector reaction mix. Digestion was carried out at 37 °C for 30 min.

Subsequently, digested DNA fragments were purified via a 1% agarose gel. Desired bands were cut out under UV light. Digested fragments were purified using the GeneJET Gel Extract Kit (Thermo Scientific) according to protocol.

Next, concentrations of digested purified vector and insert were analyzed using NanoDrop (Thermo Scientific). A vector to insert ratio of 1:3 was used to ligate both fragments for 30 min at 22 °C using T4 DNA Ligase (Thermo Scientific).

Chemically competent NEB 5-alpha *E. coli* were transformed: More precisely, bacteria were thawed on ice. Subsequently, 5 µl ligation mix was added without stirring and bacterial solution was incubated on ice for 30 min. Next, bacteria were heat-shocked in a 42 °C warm water bath for 30 seconds and placed on ice for another 5 min. 950 µl of 37 °C warm SOC medium (Thermo Scientific) were added to 50 µl of transformation mix. Recovery of transformed bacteria was carried out in shaker at 250 revolutions per minute (RPM) and 37 °C for 60 min.

Subsequently, 200 µl of transformation mix were plated out on a agarose plate containing 100 µg/ml Ampicillin. Plates were incubated at 37 °C over night. Clones were picked and used for the inoculation of 5 ml ampicillin containing lysogeny broth (LB) medium. After 12 h of incubation (140 RPM, 37 °C), cells were harvested and plasmids were purified according to the protocol of the GeneJET Plasmid Miniprep Kit.

Purified plasmids were send to GATC Biotech AG (Konstanz, Germany) for sequencing using pBR1 primer (GATC Biotech AG).

5.3.1.2 Generation of a Stable CHO-DHFR$^{(-/-)}$ Cell Line Expressing High Yields of IL-4 Mutein

CHO (DHFR$^{-/-}$) cells were transfected using electroporation: More presicely, 1-2 x 10^6 cells were harvested by centrifugation and resuspended in 4 ml ice cold culture

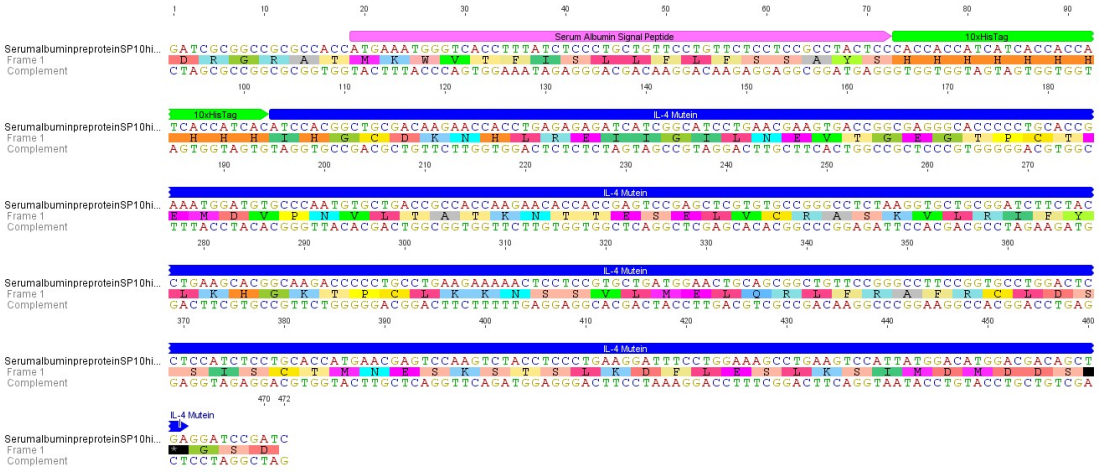

Figure 5.2: Sequence of IL-4 Mutein - Serum Albumin signal peptide (pink); His-tag (green); IL-4 Mutein sequence (blue)

medium. 400 µl of cell suspension were transferred to a electroporation cuvette. 1,5 µg of linearized expression plasmid were added to the cells. Cells were electroporated for 40 µS and 180 V. Thereafter, cells were incubated on ice for 5 min before they were transferred to a culture flask containing 5 ml CD OptiCho culture medium (Thermo scientific), . For the selection process, cells were cultured for 14 days in absence of hypoxanthine and thymidine in unsupplemented culture medium. Medium was replaced every two days.

In order to increase the yield of secreted IL-4 Mutein, several rounds of genomic amplification were performed. For this reason, cells were cultured in increasing concentrations of methotrexate (Mtx). Every 21 days, the Mtx concentration in the medium was increased. Medium was replaced with fresh medium every 3-4 days. The amplification process started with 50 nM Mtx and ended with a concentration of 2000 nM Mtx in the medium.

5.3.1.3 Recombinant Production of IL-4 Mutein

For large scale IL-4 Mutein production, CHO ($DHFR^{-/-}$) were cultured in OptiCHO medium (Thermo Scientific) at 37 °C, 8% CO_2 in a humidified atmosphere in 1450 cm^2 roller bottles until maximal density was reached.

5.3.1.4 Purification of IL-4 Mutein

Cells were removed from protein containing supernatant via centrifugation (20 min, 4 °C, 400 *G*). Supernatant was sterile filtered and purified using an ÄKTApure chromatography system (GE Healthcare Life Sciences, Freiburg, Germany): First, sterile supernatant was affinity purified using an HisTrap Excel column (GE Healthcare Life Sciences, Freiburg, Germany). Subsequently, protein containing fractions were upconcentrated using Amicron Ultra centrifugal filter Unit (Merck KGaA, Darmstadt, Germany) and applied to a HiLoad 16/600 Superdex 75pg column (GE Healthcare Life Sciences, Freiburg, Germany). IL-4 Mutein containing fractions were identified via immunoblot and upconcentrated using Amicron Ultra centrifugal filter units (Merck Millipore, Burlington, Massachusetts, USA). Next, protein containing fluid was sterile filtered using syringe filter units (Merck KGaA, Darmstadt, Germany), aliquoted and stored at -20 ℃.

5.3.2 Enzyme Linked Immunosorbent Assay

For the detection of cytokines in the serum and BAL-fluid of mice, ELISA BD Opt EIA sets were used. Maxisorp Nunc (Thermo scientific) 96 well plates were coated with capture antibody diluted 1:200 in coating buffer and incubated in sealed plates at 4 °C over night. Plates were washed three times with washing buffer. Next, unspecific binding of antibodies was prevented by addition of 200 µl blocking buffer for 2 h at room temperatre (RT). Thereafter, 50 µl of serum or BAL fluid was added to each well. Plates were incubated for 2 h at RT. Subsequently, diluted biotinylated detection antibody and streptavidin-conjugated horse radish peroxidase was added for 30 min. For detection, TMB containing substrate buffer was added. Detection process was stopped after 20-40 min with 25 µl 2 M sulfuric acid. Plates were read at 450 nm using a photometer (Tecan). The detection of immunoglobulins was performed similarly using SBA Clonotyping ELISA kits (Southern Biotech). In the case of OVA-specific immunoglobulins, OVA was coated in coating buffer.

5.3.3 Protein Analysis by Coomassie Staining and Western Blot

Supernatant of IL-4 Mutein producing cells was harvested via centrifugation. A 5x loading buffer (non-reducing) was added to the supernatant. Protein containing mix

was heated to 80 °C for 10 min, loaded on a 10% acrylamid gel and separated for 1.5 h at 150 V. Subsequently, gel was washed three times in water before either staining in colloidal coomassie solution or a western blot was performed. For the transfer of proteins from the gel to a polyvinylidenfluorid (PVDF) membrane the semi dry blotter V20 SDB was used. The electric current was limited to 500 mA. Transfer was carried out for 90 min. In order to block the unspecific binding sites of transferred proteins, the membrane was incubated for 1 h at RT in 5% non-fat dried milk powder in PBS. Subsequently, the membrane was washed three times in PBS. Next, the membrane was incubated at 4 °C over night with the primary antibody diluted in 2.5% non-fat dry milk powder in PBS. After three washing steps the membrane was incubated for 1 h at RT with the secondary antibody that is coupled with alkaline phosphatase (AP). After three more washing steps the *para*-Nitrophenylphosphate containing substrate solution was added. Enzymatic reaction was stopped via the addition of dH_2O.

5.3.4 Flow Cytometry

5.3.4.1 Cell Surface Staining of Lymphocytes Isolated from Spleen, Lymph Nodes and Lung Tissue

Single cell suspensions were prepared in FACS buffer. Thereafter, cells were pelleted (400 *G*, 5 min, 4 °C) and resuspended in Fc-block (anti-CD16/32) in order to prevent unspecific binding of immunoglobulins. Cells were incubated at 4 °C for 20 min. Next, cells were washed twice with FACS buffer and stained with 40 µl of a master mix of fluorochrome-conjugated antibodies. Cells were incubated in the dark for 30 min at 4 °C. Subsequently, cells were washed three times with FACS buffer, resuspended in 65 µl FACS buffer, filtered through a cell strainer and analyzed using BD Fortessa (Becton Dickinson, Franklin Lakes, New Jersey, USA).

5.3.4.2 Intracellular Staining for Flow Cytometry

Cells were stained for surface markers according to the described method "Surface Staining for Flow Cytometry". 200 µl Fixation/Permeabilization buffer (Thermo scientific) were added to the cells (dilute 1 part buffer concentrate with 3 parts buffer diluent). Cells were fixed in the dark over night at 4 °C. Cells were spun down at 980 *G*, at RT for 5 min. Next, fixed cells were washed twice with 200 µl permeabilization buffer (dilute 1

part concentrate with 9 parts MilliQ water prior to use). Cells were pelleted again and resuspended in an intracellular antibody mastermix in permeabilization buffer. Cells were incubated with master mix for 1 h at RT in the dark. Subsequently, cells were washed twice with PBS, resuspended in 50 µl PBS for FACS analysis.

5.3.5 BCA Assay

A Pierce BCA Protein assay kit was used to determine the concentration of the produced protein. Briefly, the kit combines the well known reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) using a reagent containing bicinchoninic acid (BCA). The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range. Using a BSA stock solution, a dilution series of BSA from 0-2000 µg/ml was produced. This dilution series and the solution containing the protein was measured in a photometer (Tecan) at 562 nm.

5.3.6 Endotoxin Quantification Assay

For the detection of possible endotoxin-contaminations, the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) was used according to protocol. Briefly, the assay works as follows: Endotoxins catalyze the activation of a proenzyme in a modified Limulus Amebocyte Lysate (LAL). The activated proenzyme catalyzes the splitting of *p*-nitroaniline from a colorless substrate. After stopping of the reaction, *p*-nitroaniline is can be detected photometrically at 405 nm.

5.3.7 Synthesis and Charaterization of PLGA-PEG-PLGA Triblock Copolymer

5.3.7.1 Synthesis of PLGA-PEG-PLGA Triblock Copolymer

The PLGA-PEG-PLGA triblock copolymer was synthesized according to Qiao, D. Chen, Ma, et al., 2005. Polyethylene glycol 1500 was dried in a three-necked round bottom flask under vacuum and gentle stirring at 120 °C for 2 h. Against an argon stream, 16,6 g lactide and 0,89 g glycolide were added to the PEG1500. After 30 min

40 µl of TIN(II)-ethylhexanoate were added against an argon stream to the reaction. Temperature was increased to 150 °C and the polymerization reaction was carried out for 16 h under gentle stirring. The resulting polymer was dissolved in 25 ml acetonitrile. When completely dissolved, 150 ml MilliQ water was added. After complete dissolution, the polymer solution was heated to 80 °C. Polymer precipitated and educt-containing supernatant was removed. Polymer was dissolved two more times in water and precipitated. Finally, polymer was aliquoted and freeze dried.

5.3.7.2 Tube Inversion Method for the Determination of Gelling Temperature

2 ml reaction tubes were filled with 1 ml of differently concentrated polymer solutions. Reaction tubes were placed in a heating block. Temperature was increased by 1 °C every 5 min. Before each temperature increase, tubes were inversed. The sol-gel conversion was determined by the lowest temperature that induced gel formation and prevented the polymer solution from flowing from the bottom of the tube after inversion. The precipitation temperature was determined as the lowest temperature at which the polymer-solution started flowing from the bottom of the tube after the inversion again.

5.3.7.3 *In vitro* Protein Release Assay

In order to assess the protein release characteristics of the polymer, an *in vitro* release assay was performed: A 20% polymer in PBS solution was loaded with IL-4 Mutein, gelled in a 2 ml reaction tube, overlayered with PBS and incubated at 37 °C with gentle shaking. The PBS was replaced with fresh PBS every 24 h. The released IL-4 Mutein in the PBS was assessed by IL-4 ELISA.

5.4 Practical Contribution to Particular Chapters

I synthesized the PLGA-PEG-PLGA polymer and recombinantly produced IL-4 Mutein for all experiments in our laboratories. All following *in vitro* analyses we performed by me in our facilities.

The animal experiments "Effect of IL-4 Mutein on the Alum Mediated Sensitization to the Model Allergen Ovalbumin" and "Murine Model of Allergen-Specific Immunotherapy" (5.1.3.1 and 5.1.3.3) and associated analyses were carried out in the German Mouse Clinic by Dr. Antonio Aguilar-Pimentel.

I performed all other animal experiments and following analyses in our institute.

5.5 Data Analysis and Statistics

Analysis of data was performed using GraphPad Prism or Microsoft Excel. Significance of Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively. Results were considered significant as $^{*}p \geq 0.05$, $^{**}p \geq 0.01$, and $^{***}p \geq 0.001$.

6

Material

6.1 Buffers and Media

ACK lysis buffer

8,26 g NH_4Cl, 1 g $KHCO_3$, 37,2 mg Na_2EDTA, ddH_2O, pH 7,4

Anesthesia

100 µl ketamine, 1 ml xylazin, 8,9 ml PBS

Medium for BAL

PBS, 5% FCS (V/V)

AP-Detection buffer

10mM Tris, 10 mM $MgCL_2$ x $6H_2O$ ddH_2O, pH 9,5

CD OptiCHO medium

CD OptiCHO AGT medium [freeze dried], ddH_2O

Complete RPMI 1640

440 ml RPMI1640, 50 ml FCS, 5 ml Glutamin (x100), 5 ml PenStrep (x100)

Colloidal Coomassie blue

10% $(NH_4)_2SO_4$, 0,1% Coomassie G-250, 3% orthophosphoric acid, 20% ethanol

Galatti buffer

0,2 M citric acid monohydrate (pH 4,0)

Detection buffer HRP

15 ml Galatti buffer (pH 4,0), 3,3 mg ABTS, 18% H_2O_2

Elution buffer

300 mM imidazole, PBS, pH 7,4

TAE buffer (50x)

2 M Tris, 50 mM EDTA, ddH_2O, pH 8,5

Transfer buffer (semi dry)

25 mM Tris, 19,2 mM Glycin, 20% isopropanol, ddH_2O, pH 8,3

Tris buffer

1 mM Tris, ddH_2O, pH 7,5 or pH 9,0

Tris-Tricin anode buffer (x5)

0,2 M Tris, ddH_2O, pH 8,9

Tris-Tricin kathode buffer (x5)

0,5 M Tris, 0,5 M Tricin, 0,5% SDS, ddH_2O, pH 8,6

Tris-Tricin gel buffer (x3)

3 M Tris, 0,3% SDS, ddH_2O, pH 8,45

Tris-Tricin sample buffer (x4)

0,2 M Tris, 48% glycerol, 16% SDS, 0,4 M DTT, 0,04% CXoomassie-Brilliant Blue R-250, ddH_2O, pH 6,8

ELISA washing buffer

PBS, 0,05% Tween20

ELISA coating buffer

5,2 g $NaHCO_3$, 1,78 g Na_2PO_4, on 500 ml dH_2O. pH 9,5

ELISA blocking buffer

PBS, 10% FCS

ELISA substrate buffer

0,2 M citric acid monohydrate (pH 4); add 1:200 TMB stock solution (24 mg/ml) and 1:2000 30% H_2O_2

ELISA stop solution

2 M H_2SO_4

6.2 Material for Cell Biology

Substance	Manufacturer
anti-IFNγ	Biolegend
anti-CD3	BD Pharmingen
anti-CD28	BD Pharmingen
DMEM	Invitrogen
FCS	PAA
Glutamine	Invitrogen
Interleukin-2	ImmunoTools
Interleukin-4	PreproTech
Methotrexate	Sigma Aldrich
Neomycin	Thermo Scientific
Non-essential amino acids	Invitrogen
Pen/Strep	Invitrogen
RPMI1640	Invitrogen
Sodium pyruvate	Invitrogen
TGF-β	PreproTech
Trypanblue	Sigma Aldrich
Trypsin-EDTA	Invitrogen
β-Mercaptoethanol	Sigma Aldrich
Zeocin	Invitrogen

Table 6.1: Material for cell biology

6.3 Material for Molecular Biology and Protein Chemistry

Substance	Manufacturer
ABTS	AppliChem
BCIP	AppliChem
Bovine serum albumin	Sigma
Acrylamid	ROTH
Agarose	Fluka Chemicals
Ampicillin	Sigma
APS	AppliChem
Coomassie Brilliant Blue R-250	AppliChem
DTT	AppliChem
Glycerol	Fluka Chemicals
Glycin	AppliChem
Imidazole	AppliChem
NBT	AppliChem
N,N,N,N-Tetramethylethylenediamide	Serva
PBS	Invitrogen
SDS	AppliChem
TMB	Fluka Chemicals
Tris	AppliChem
NotI	Thermo Scientific
BamH1	Thermo Scientific
Ligase	Thermo Scientific
Alkaline phosphatase	Thermo Scientific
Fast Sigest Green buffer	Thermo Scientific
Page ruler prestained plus	Thermo Scientific
DNA ladder gene rulter mix	Thermo Scientific

Table 6.2: Material for molecular biology and protein chemistry

Kit	Manufacturer
Pierce BCA assay	Thermo Scientific
PPierce LAL Chromogenic Endotoxin Quantitation Kit	Thermo Scientific
Plasmid preparation kit	Thermo Scientific
Gel extraction kit	Thermo Scientific
Naive CD4 T cell isolation kit	Miltenyi
CellTiter-BLue cell viability assay	Promega

Table 6.3: Kits for molecular biology and protein chemistry

6.4 Material for Animal Experiments

Substance	Manufacturer
Imject Alum	Sigma Aldrich
Ketamine	WDT
OVA V	Sigma
Isofluorane	Baxter
PBS	Invitrogen
Sevofluorane	Baxter
Xylacine	aniMedica

Table 6.4: Material for animal experiments

6.5 Material for ELISA

Name	Manufacturer
IFNγ	BD OptEIA™
IL-4	BD OptEIA™
IL-5	BD OptEIA™
IL-6	BD OptEIA™
IL-10	BD OptEIA™
IL-13	Invitrogen Cytoset
IL-17	Invitrogen Cytoset
Total IgG1	BD Pharmingen
Total IgE	BD Pharmingen
OVA IgG1	BD Pharmingen
OVA IgG2a	BD Pharmingen
OVA IgG2b	BD Pharmingen
OVA IgE	BD Pharmingen

Table 6.5: ELISA kits

6.6 Material for the Synthesis and Characterization of PLGA-PEG-PLGA Triblock Copolymer

Name	Manufacturer
Glycolide	Sigma
Acetonitrile	ROTH
Lactide	abcr
PEG1500	Sigma
Tin(II) 2-ethylhexanoate	Sigma

Table 6.6: Material for the synthesis and characterization of PLGA-PEG-PLGA triblock copolymer

6.7 Material for Flow Cytometry

Marker	Fluorochrome	Clone	Manufacturer
CD3ϵ	FITC	145-2C11	BD Pharmingen
CD3ϵ	BV785	17A2	BioLegend
CD3ϵ	AF700	17A2	BioLegend
CD4	BV711	RM4-5	BioLegend
CD4	PerCP-Cy5.5	RM4-5	BD Pharmingen
CD45	APC eF780	30-F11	eBiosciences
CD45	PerCP-Cy5.5	30-F11	BioLegend
F4/80	APC-e780	BM8	eBiosciences
FoxP3	PerCP-Cy5.5	FJK-16s	eBiosciences
GATA3	eF660	TWAJ	eBiosciences
Ki-67	FITC	B56	BD Pharmingen
RorγT	PE	AFKJS-9	eBiosciences
ST2	APC	RMST2-2	Affymetrix
ST2	BV421	DIH9	MD Bioproducts
Live/dead aqua fixabel cell stain	405 nm	—	Thermo Fisher

Table 6.7: Antibodies for FACS analysis of lymphocytes isolated from lung tissue

Marker	Fluorochrome	Clone	Manufacturer
CD3ϵ	FITC	145-2C11	BD Pharmingen
CD3ϵ	BV785	17A2	BioLegend
CD3ϵ	AF700	17A2	BioLegend
CD4	BV711	RM4-5	BioLegend
CD4	PerCP-Cy5.5	RM4-5	BD Pharmingen
CD8	PE	53-6.7	BD Pharmingen
CD8	FITC	53-6.7	BD Pharmingen
CD11b	BV711	M1/70	BioLegend
CD11b	eF450	M1/70	eBiosciences
CD11c	PE-Cy7	N418	Biolegend
CD19	Pacific Blue	6D5	BioLegend
CD25	PE	PC61.5	eBiosciences
CD45	APC eF780	30-F11	eBiosciences
CD45	PerCP-Cy5.5	30-F11	BioLegend
CD117	FITC	2B8	BioLegend
CD206	PE-Dazzle594	C068C2	BioLegend
F4/80	APC-e780	BM8	eBiosciences
NK1.1	APC	PK136	eBiosciences
ST2	APC	RMST2-2	affymetrix
ST2	BV421	DIH9	MD Bioproducts
Ly-6G	BV570	1A8	BioLegend
Ly-6G	Pacific Blue	1A8	Biolegend
Ly-6C	AF700	AL-21	BD Pharmingen
Siglec-F	PE-Dazzle594	E50-2440	BD Pharmingen
Siglec-F	PE	E50-2440	BD Pharmingen
Live/dead aqua fixabel cell stain	405 nm	—	Thermo Fisher

Table 6.8: Antibodies for FACS analysis of BAL cells

6.8 General Material and Equipment

Consumable	Type	Manufacturer
96 well plate	Cell culture	Nunclon delta surface
96 well plate	MaxiSorp	Thermo Scientific
Cell strainer	70 µm	Greiner Bio-One
FACS tube	Microtube	Greiner Bio-One
Serological pipettes	1 ml, 2 ml, 5 ml, 10 ml, 25 ml	Sarstedt
Reaction tube	0,2 ml, 0,5 ml, 1,5 ml, 2 ml, 5 ml	Sarstedt
Roller bottle	1700 cm_2	Corning
Flat bottom tube	6 ml	Sarstedt
Sterile filter	0,22 µm	Merck Millipore
Pipette tips	10 µl, 200 µl, 300 µl, 1000 µl	Sarstedt
Tubes	15 ml, 50 ml	Sarstedt
Serum tube	Microvette	Sarstedt

Table 6.9: Consumables

Software	Software publisher
Geneious R 8.1.5	2005-2015 Biomatters
i-control	Microplate reader software, TECAN
EndNote X8.2	Clarivate Analytics
GraphPad Prism 5.04	GraphPad Software inc.
ImageJ 1.48v	National Institutes of Health, USA
TEXnic center 2.02	TEXnic center team
PDF-XChange editor plus	Tracker Software Products (Canada) Ltd.
MestReNova	Mestrelab Reseach
FlowJo V10	FlowJo, LLC, 2013-2018
BD FACSDIVA	BD Biosciences

Table 6.10: Software

Equipment	type	Manufacturer
Flow cytometer for cell counting	Accuri C6	BD Pharmingen
Flow Cytometer	LSR FOrtessa	BD Pharmingen
FPLC	ÄKTA Pure	GE Healthcare Life Sciences
FPLC	ÄKTA Prime	GE Healthcare Life Sciences
Centrifuge	5424R	Eppendorf
Centrifuge	5810R	Eppendorf
CO_2 incubator	Galaxy 170 S	New Brunswick
Freezer (-20 °C)	Premium Nofrost	Liebherr
Freezer (-80 °C)	V570 HEF	New Brunswick
Fridge (4 °C)	MediLine	Liebherr
Heating block	RCT basic	IKA
Microscope	Modell MS 5	Leica
Nebulizer	PARI Boy SX	PARI
Neubaur counting chamber	Haemacytometer-CD doppelt	Assistent
PCR cycler	Nexus gradient	Eppendorf
pH meter	pHenomenal 1100L	VWR
Photometer	infinite m200 pro	Tecan
Pipettes	pePETTE	Peqlab
Pipettor	accu-jet pro	Brand Industries
Vortex	Vortex genie	Scientific industries
Microplate washer	Hydrospeed Tecan	Tecan
Freezy dryer	Alpha 1-2D plus	Christ
Shaking incubator	Innova 42	New Brunswick
Microvolume spectral photometer	Nanodrop	Thermo Scientific
Vacuum pump	LVS 302Z	Ilmvac
Oil pump	RZ2.5	Vacubrand
Agarose gel chamber	PerfectBlue	Peqlab
Semi dry blotter	V20 SDB	Peqlab
Protein electrophoresis chamber	Hoefer 250SE	Hoefer
Power supply	Peqpower	Peqlab
Thermomixer	Thermomixer C	Eppendorf

Table 6.11: Laboratory equipment

7

Results

7.1 Production and Characterization of Recombinant IL-4 Mutein

The foundation of the embraced project was the recombinant production of murine IL-4 Mutein and the analysis of its IL-4 receptor antagonizing abilities.

7.1.1 Expression of IL-4 Mutein in CHO Cells

Different expression systems were analyzed for their suitability to recombinantly produce IL-4 Mutein. Expression in E. coli (BL21) transformed with the vector Pet22b$^+$ resulted in expression of IL-4 Mutein with very low biological activity (only 12.3% of total expressed protein was detected using an ELISA-kit that only detects correctly folded IL-4). Protein expression in human embryonic kidney cells (HEK293) that were stably transfected with pcDNA3.1 did not result in an adequate protein yield for *in vivo* experiments. Expression in the insect cell lines Sf9 and Sf21 was not successful due to a faulty secretion of IL-4 Mutein from the cells that resulted in an intracellular aggregation.

Ultimately, IL-4 Mutein was expressed in dihydrofolate reductase deficient (DHFR$^{-/-}$) chinese hamster ovary (CHO) cells. The coding sequence of IL-4 Mutein was cloned into a pIRES-DHFR vector system in which the gene of interest is coupled via an internal ribosomal entry site (pIRES) to the selection marker DHFR (Figure 7.1a). In order to obtain higher protein yields, transfected CHO cells were slowly adapted to increasing methotrexate concentrations. Cultivation of CHO cells in the absence of

methotrexate resulted in a protein yield of 0.5 mg/L. The protein yield could be increased to 8.5 mg/L in cells that were adapted to cultivation medium containing 2000 nM methotrexate (Figure 7.1b). The His-tagged protein was purified via $Ni2^{+}$-affinity chromatography and size exclusion chromatography. The resulting protein ran at approximately 22 kDa on a SDS page gel (Figure 7.1c) and could be detected via an anti-IL-4 antibody in a Western blot (Figure 7.1d).

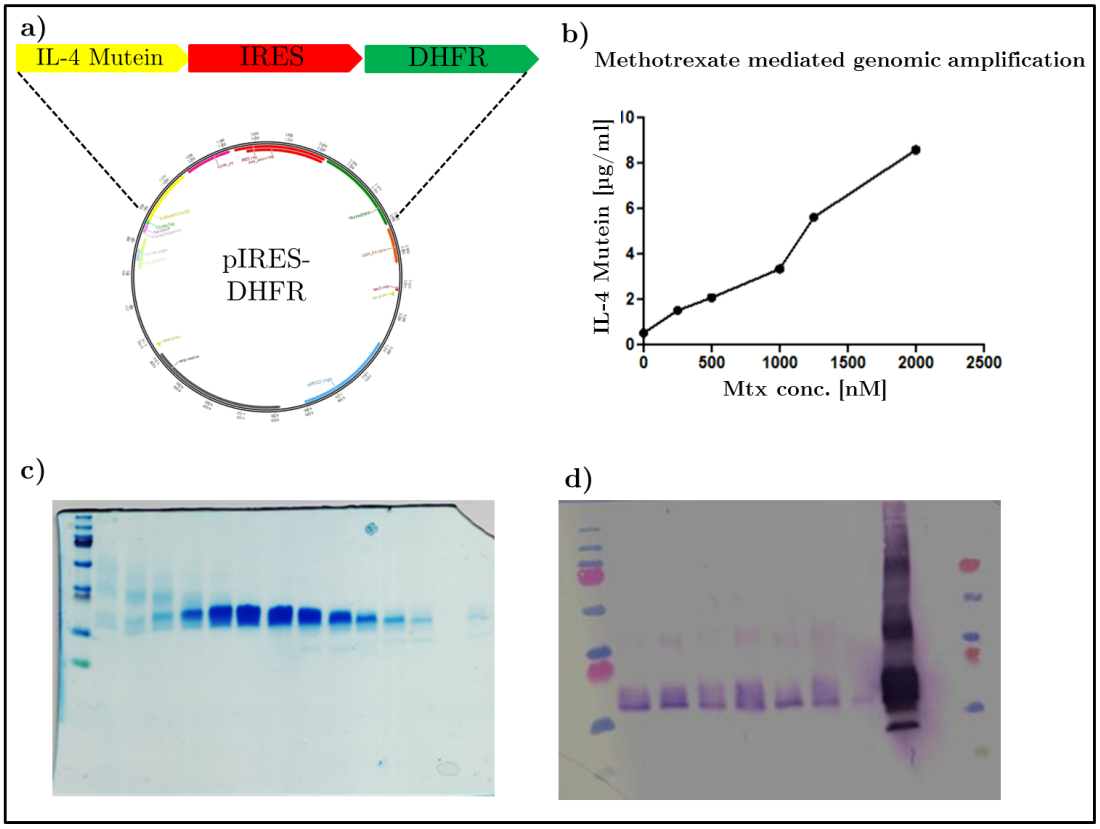

Figure 7.1: Recombinant expression of IL-4 Mutein in CHO cells - a) pIRES-DHFR vector with IL-4 Mutein coupled to DHFR via an IRES; b) Increasing methotrexate concentrations result in an increase of IL-4 mutein production c) Coomassie gel of affinity and size exclusion-purified IL-4 Mutein; d) Western blot of affinity and size exclusion-purified IL-4 Mutein.

7.1.2 Biologic Activity of IL-4 Mutein

The biological activity of IL-4 Mutein was tested in different cellular assays. In order to analyze its IL-4 antagonizing effect on primary T cells, naive $CD4^{+}$ T cells from 4get

reporter mice were used. This knock-in mouse line is characterized by a bicistronic IL-4 mRNA which carries a viral IRES and the coding sequence for enhanced green fluorescent protein (eGFP) (Mohrs et al., 2001). Cultivation of naive $CD4^+$ lymphocytes from 4get mice in the presence of IL-4 resulted in a strong TH2 differentiation. Approximately 45% of T cells were eGFP positive and therefore IL-4-producing TH2 cells. Addition of a 100 fold excess of IL-4 Mutein to this TH2-polarizing medium led to a complete suppression of IL-4 positive T cells (Figure 7.2a).

In a second cellular assay, naive $CD4^+$ T cells from reporter mice expressing red fluorescent protein (RFP) coupled with FoxP3 expression were used. Cultivation of naive $CD4^+$ T cells in the presence of IL-2 and TGF-β resulted in a strong Treg polarization that was characterized by an increase of FoxP3 positive cells. Addition of 5 ng/ml IL-4 to this Treg-polarizing medium led to the suppression of FoxP3 expression. Addition of a 100 fold IL-4 Mutein over IL-4 excess, however, could rescue the IL-4-suppressed FoxP3 expression (Figure 7.2b).

To further precisely quantify the IL-4-antagonizing effect of IL-4 Mutein, a lymphocyte cytotoxic T lymphocyte cell line (CTLL2) was used. Survival of this cell line is dependent on the presence of either IL-2 or IL-4. In order to determine the IC_{50} value of CHO derived IL-4 Mutein, cells were cultured in presence of 5 ng/ml IL-4 and increasing IL-4 Mutein concentrations. A 25 fold IL-4 Mutein to IL-4 excess was calculated as IC_{50} value (Figures 7.2c and 7.2d).

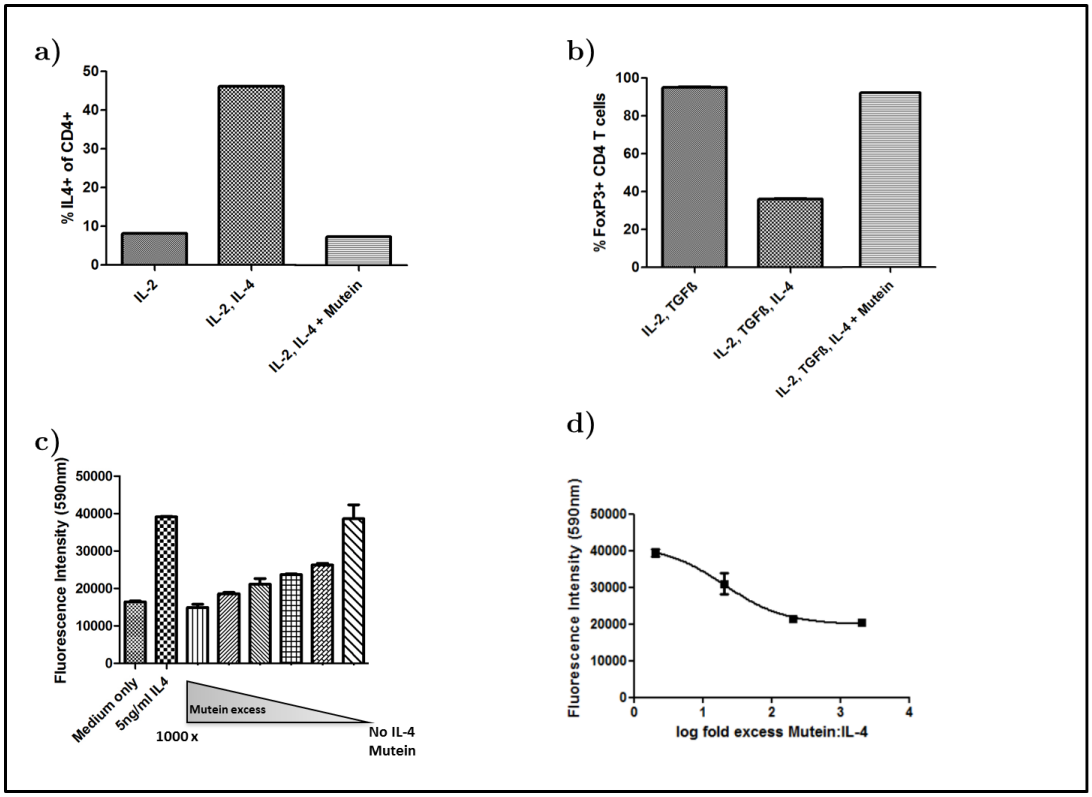

Figure 7.2: Biologic activity of IL-4 Mutein *in vitro* - a) Effect of IL-4 Mutein on IL-4-mediated TH2 polarizaion of naive T cells b) Impact of IL-4 Mutein on IL-4-induced Treg suppression c) and d) Quantification of the IC_{50}value of IL-4 Mutein in a CTLL2 assay.

7.2 PLGA-PEG-PLGA Triblock Copolymer-based Hydrogel as Delivery Matrix for Allergens and Biologicals in AIT

A PLGA-PEG-PLGA polymer-based hydrogel was synthesized and characterized in detail, as its thermosensitive gelling behaviour and its *in vivo* protein release characteristics are promising abilities for the delivery of allergens during AIT and biologicals with short serum half life.

7.2.1 Chemical Characterization of PLGA-PEG-PLGA Copolymer

The molecular weight distribution of the in-house synthesized polymer was determined via gel permeation chromatography (Figure 7.3a) The obtained number average molar mass (M_n) was 5669 g/mol, the mass average molar mass (M_w) could be determined to 7118 g/mol which results in a polydispersity index (D) of 1,26. The Z average molar mass (M_z) was 8590 g/mol. Further, the gelling temperature of differently concentrated polymer solutions was assessed (Figure 7.3b). A 20% polymer in PBS solution (w/v) showed ideal gelling characteristics for *in vivo* applications as the solution was liquid at room temperature and became solid at 32 °C. A further temperature increase to 46 °C resulted in a liquidation of the formed gel. An ^{1}H NMR analysis was performed to screen the synthesized polymer for possible contaminations and to determine the lactide to glycolide ratio (Figure 7.3c). As only the expected peaks could be measured, no signs of impurities could be found in the polymer. The lactide:glycolide ration was calculated to be approximately 19:1.

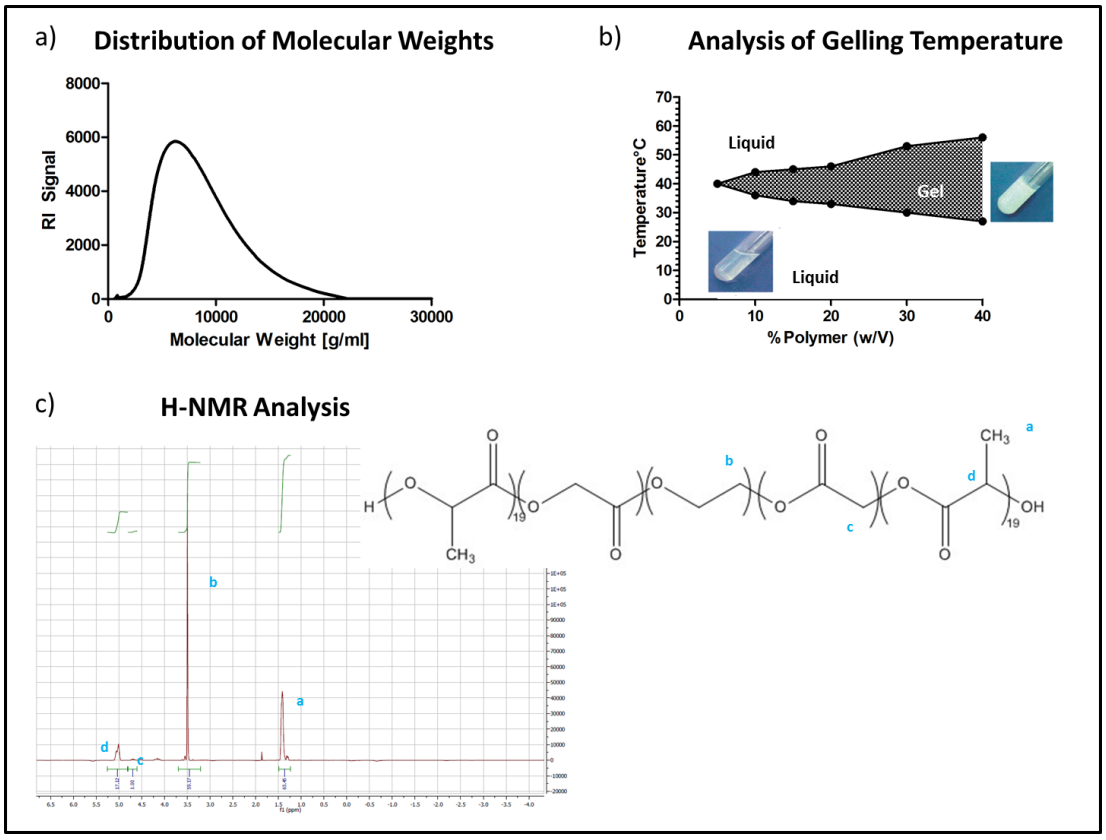

Figure 7.3: Chemical characterization of PLGA-PEG-PLGA triblock copolymer - a) Molecular weight distribution of polymer according to gel permeation chromatography; b) Gelling temperature of polymer in depending on polymer concentration; c) H-NMR analyis of polymer.

7.2.2 PLGA-PEG-PLGA Triblock Copoymer-based Hydrogel as Delivery Matrix for Proteins *in vivo*

The protein release characteristics of a 20% (w/v) polymer solution was assessed in an *in vitro* release assay. After an early release burst in the first 24 hours, the gel depot continued to release a stable amount of IL-4 Mutein over 5 days. As a next step, the release characteristics of the polymer were tested *in vivo* (Figure 7.4b): Mice were s.c. injected with an IL-4 Mutein loaded 20% polymer solution or IL-4 Mutein in PBS. 24 hours after the injection, the serum concentration of IL-4 Mutein was measured via ELISA. The mice that had received IL-4 Mutein in hydrogel showed a significantly higher serum concentration when compared to mice that had received IL-4 Mutein in PBS.

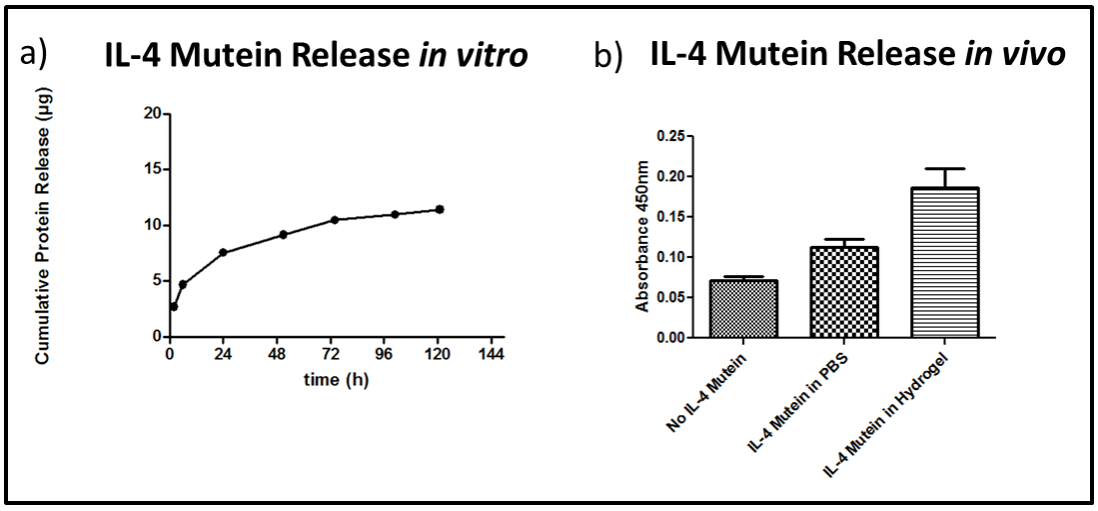

Figure 7.4: Protein release characteristics of PLGA-PEG-PLGA triblock copolymer - a) Cumulative release of IL-4 Mutein *in vitro* b) Release of IL-4 Mutein from polymer *in vivo*

Next, the polymer was used as delivery matrix for OVA in the course of a murine model for OVA-specific AIT. The positive control mice that were sensitized with OVA complexed with alum and challenged with nebulized OVA showed high numbers of infiltrated cells (amongst others eosinophils and lymphocytes) in the BAL. These cell numbers were strongly decreased when an OVA-specific AIT was performed. Mice that were sensitized with OVA/alum, challenged with nebulized OVA and received an OVA-specific AIT showed significantly lower BAL cell numbers (Figure 7.5). As there were

no differences regarding BAL cell numbers between the mice that received the OVA in the polymer solution and the mice that were injected with OVA in PBS, the polymer solution respresents a promising delivery matrix for allergens that could potentially be loaded with biologicals or other adjuvant substances.

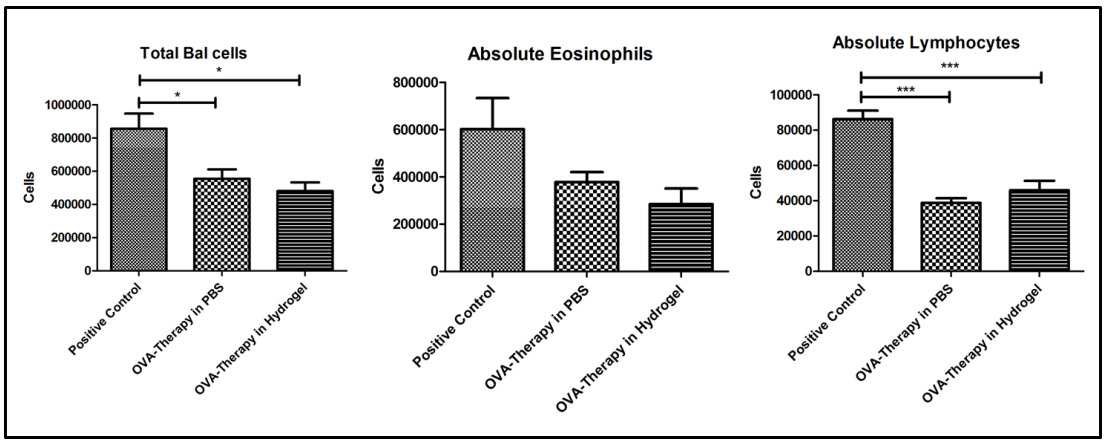

Figure 7.5: Analysis of BAL cells after AIT using PLGA-PEG-PLGA copolymer as delivery matrix for OVA in the course of AIT - Data are given as means +/- SEM (n=5). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.3 IL-4 Mutein Prevents Alum-Mediated Sensitization to Ovalbumin *in vivo*

The following experiment was performed in cooperation with Dr. Antonio Aguilar Pimentel in the facilities of the GMC.

Via daily intraperitoneal administrations of 100 µg IL-4 Mutein on eight consecutive days before and after i.p. OVA/alum sensitization, the prophylactic effect of IL-4 Mutein treatment on the alum-mediated sensitization was analyzed (Figure 7.6). Mice were bled on day 8 after sensitization. Serum was analyzed for immunoglobulin and cytokine levels.

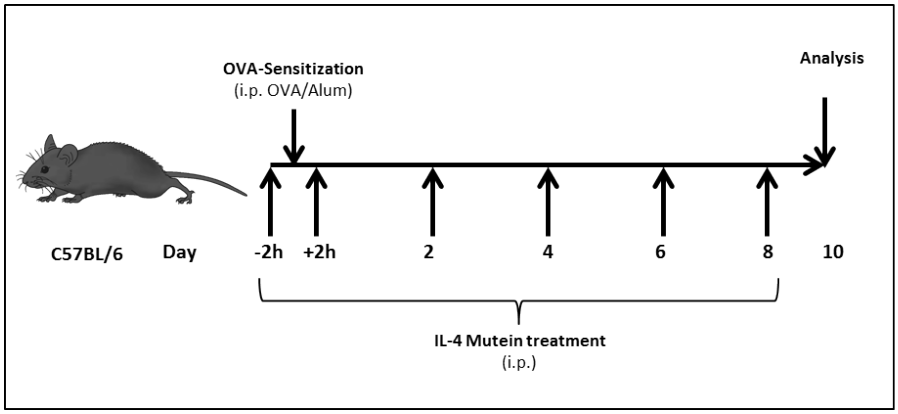

Figure 7.6: Murine model "Prophylactic IL-4 Mutein treatment during OVA/alum sensitization" - Mice (C57BL/6J, n=15) were treated as follows: Positive control mice were i.p. injected with OVA/alum; negative Control mice received alum with PBS i.p.; Mutein therapy mice were injected with OVA/alum and additionally received one i.p. injection IL-4 Mutein before and eight injections after OVA/alum injection.

As expected from earlier studies (Hahn et al., 2003), i.p. sensitization with OVA/alum resulted in increased total IgE and OVA-specific IgE levels. These increases could be suppressed by prophlyactic administration of IL-4 Mutein. Mice from the positive control group that were sensitized with OVA complexed with alum showed significantly higher concentrations of IgG1 than their negative control counterparts which where sensitized using alum loaded with PBS. IL-4 Mutein treated mice showed an even higher level of IgG1 in the serum. Further, the administration of IL-4 Mutein led to an increase of different immunoglobulin subtypes that were not affected by the OVA/alum sensitization. The total levels of IgG2a, IgG2b, IgG3 and IgM were significantly increased after IL-4 Mutein therapy (Figure 7.7).

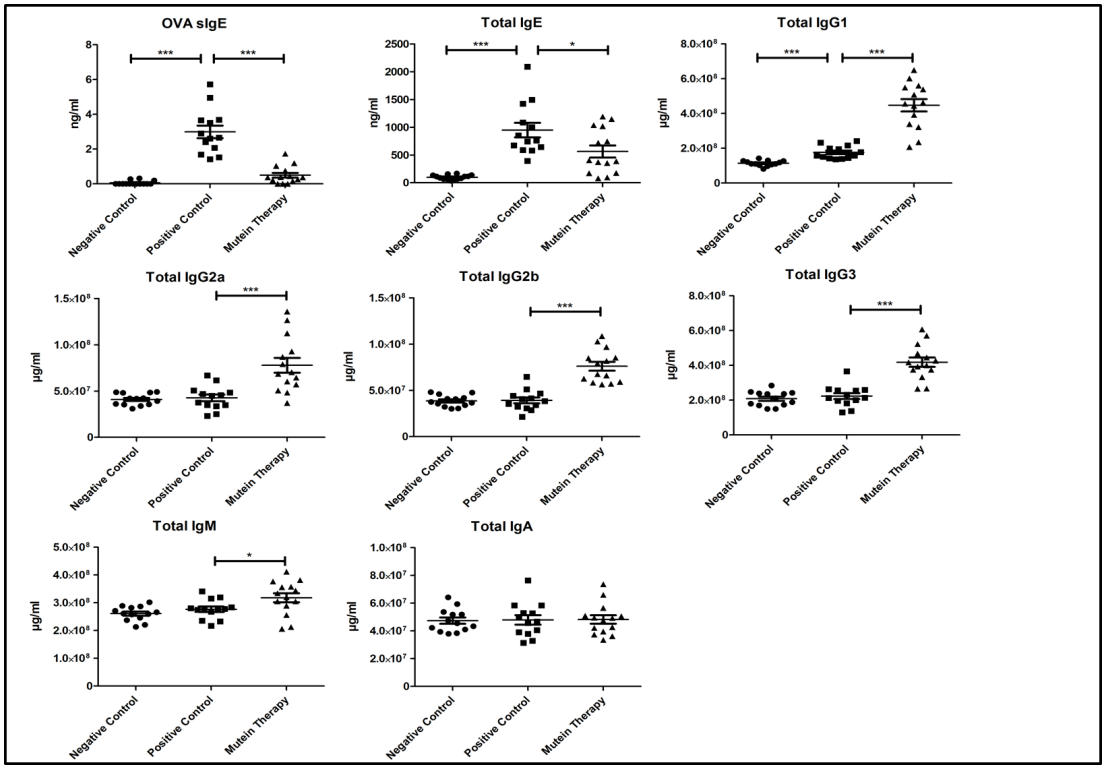

Figure 7.7: Effect of preventive IL-4 Mutein treatment on immunoglobulin levels during OVA/alum sensitization - Data are given as means +/- SEM (n=15). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

The prophylactic treatment with IL-4 Mutein also induced several changes on cytokine levels in the serum. The detected non-physiological level of IL-4 was surely caused by remaining IL-4 Mutein in the organism that could be detected via IL-4 ELISA. The IL-5 concentration in the serum of sensitized mice was significantly increased compared to their untreated counterparts. This increase could be entirely abolished by IL-4 Mutein administration. Other cytokines that showed a decreased concentration in sensitized mice (IL-1β, IL-6 and TNFα) showed concentrations comparable to non-sensitized mice after IL-4 Mutein treatment. Solely the reduction of keratinocyte chemoattractant/human growth-regulated oncogene (KC/GRO) in sensitized mice was not reversed by IL-4 Mutein treatment. The serum abundance of IL-2, IL-10, IL-12 and Interferonγ (IFN-γ) did not vary between the differently treated groups (Figure 7.8).

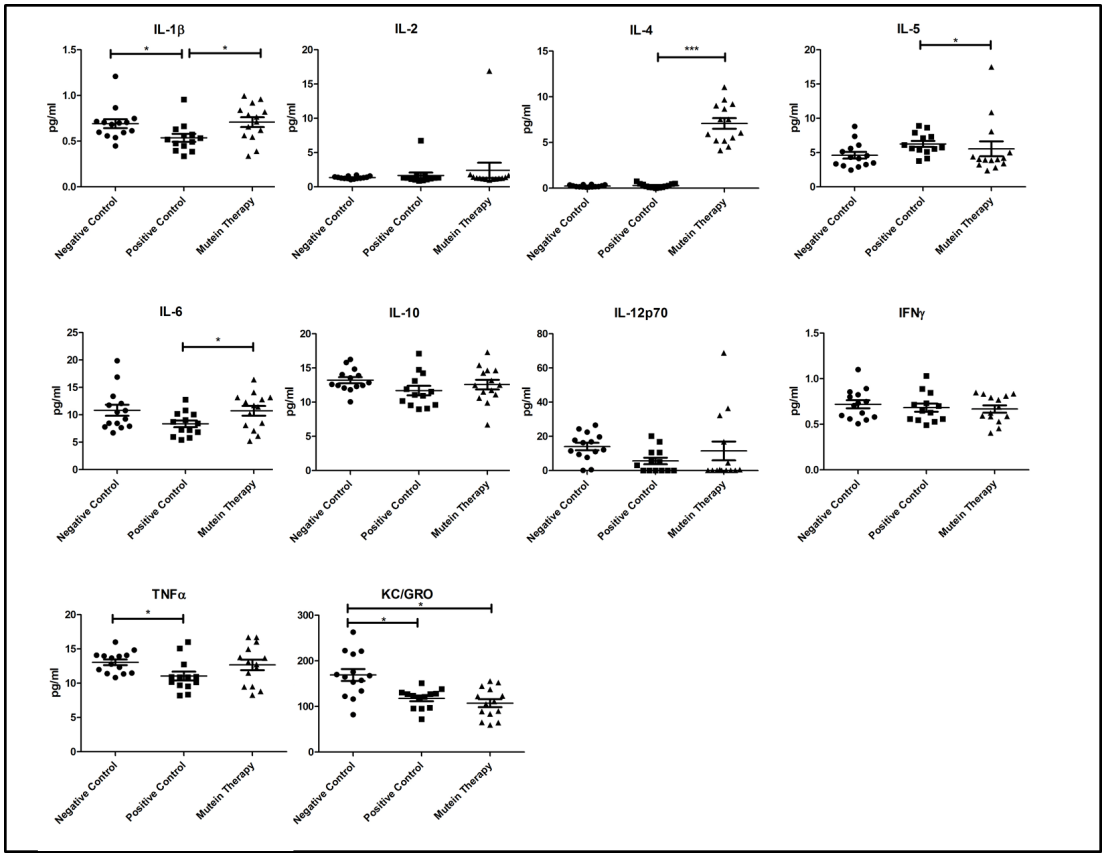

Figure 7.8: Effect of preventive IL-4 Mutein treatment on cytokine levels after OVA/alum sensitization - Data are given as means +/- SEM (n=15). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.4 IL-4 Mutein Suppresses House Dust Mite-Mediated Lung Inflammation

A model of house dust mite induced allergic lung inflammation was used to assess the effect of IL-4 Mutein treatment on the acute phase of lung inflammation. Mice were intranasally (i.n.) sensitized on day 1 with house dust mite extract. Seven days later, a 5-day i.n. challenge phase was performed. During this challenge phase, mice received daily i.p. injections of 100µg IL-4 Mutein 30 minutes before each challenge. Mice were euthanized 72 h after the last challenge (Figure 7.9). Mice in the positive control group, that were sensitized and challenged with house dust mite extract showed a strong

increase in total IgE levels and a striking rise of total BAL cells when compared to negative control mice that were only treated with PBS. Both HDM-caused elevations, that are linked with allergic lung inflammation, could be strongly reduced by IL-4 Mutein treatment (Figure 7.10).

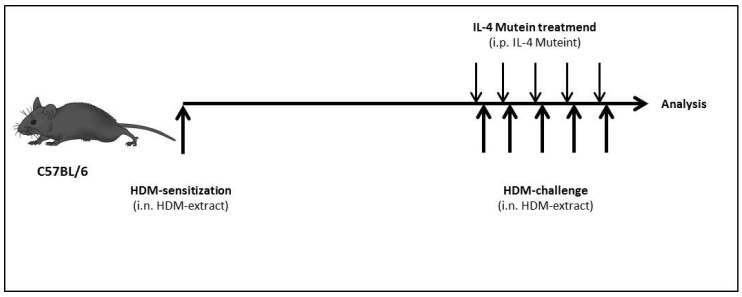

Figure 7.9: Murine model "Effect of IL-4 Mutein on house dust mite-induced lung inflammation" - Mice (C57BL/6J, n=5) were treated as follows: Positive control mice were intranasally sensitized and challenged with house dust mite extract; negative control mice were sensitized and challenged with PBS; IL-4 Mutein treated mice were sensitized and challenged with HDM extract and additionally i.p. injected with IL-4 Mutein before every i.n. challenge.

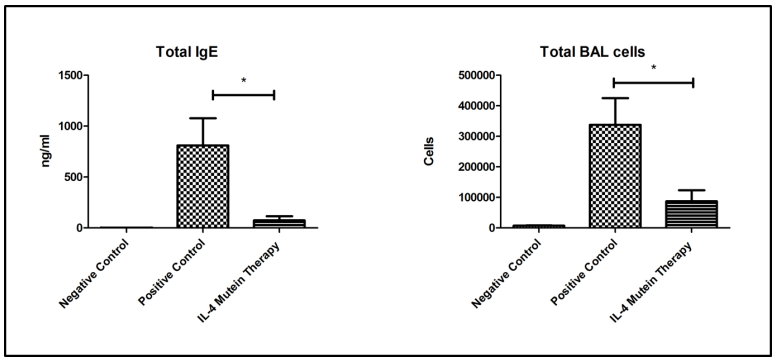

Figure 7.10: Impact of IL-4 Mutein on the murine model "House dust mite-induced lung inflammation" - Data are given as means +/- SEM (n=5). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.5 Effect of IL-4 Mutein on a Murine Model of Allergen-Specific Immunotherapy

The following experiment was conducted in cooperation with Dr. Antonio Aguilar Pimentel in the facilities of the GMC.

As a next step, the potential therapeutic benefit of the combination of IL-4 Mutein with AIT was analyzed. For this reason, a widely used murine model of OVA-specific allergen-specific immunotherapy (AIT) was chosen (Figure 7.11). As expected, mice from the positive control group that were sensitized with OVA complexed with alum and challenged with nebulized OVA showed a significantly higher number of cells in the bronchoalveolar lavage (BAL) compared to non-sensitized and challenged mice from the negative control group. The vast majority of infiltrated cells was identified as eosinophils. However, also lymphocytes were increased in the positive control group. In accordance with the literature, OVA-specific AIT could significantly reduce the number of total cells in the BAL of mice from the OVA therapy group that received OVA-specific AIT. However, the cellular composition of BAL cells was almost not affected by AIT. Solely the fraction of neutrophils was slightly increased when compared with mice from the positive control group. The addition of IL-4 Mutein to AIT did not result in any changes regarding BAL cell number or composition when compared to mice that received OVA-specific AIT alone. Hence, mice that received AIT in combination with IL-4 Mutein did not show any changes on BAL cell level when compared to mice that had received the AIT alone (Figure 7.12).

Next, the cytokine pattern in the BAL of mice from AIT and control groups was analyzed. Mice from the positive control group that were sensitized and challenged showed different cytokine levels than their non-sensitized counterparts. The cytokines IL-4, IL-5, IFNγ, TNFα, IL-6, IL-10 and IL-1β were significantly increased. AIT led to a decrease of IFNγ, IL-6, IL-10 and IL-1β. TNFα levels, however, were increased after AIT. The additional administration of IL-4 Mutein resulted in a further reduction of IL-6. The remaining cytokines were not affected by IL-4 Mutein treatment (Figure 7.13).

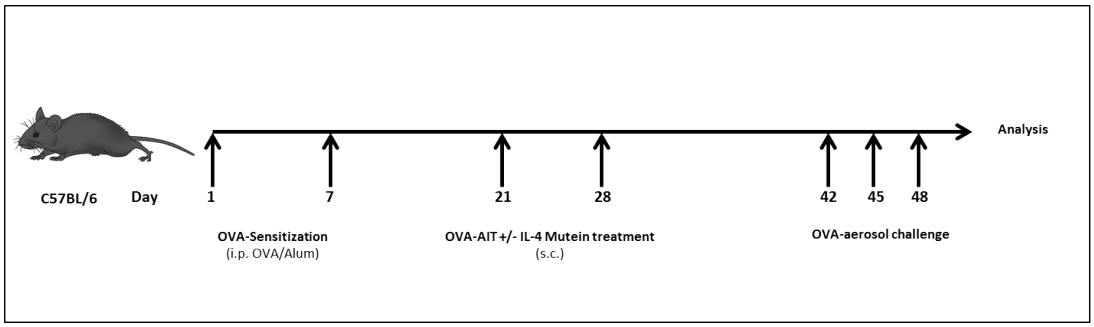

Figure 7.11: Murine model "Allergen-specific immunotherapy" - Mice (C57BL/6J, n=15) were treated as follows: Mice in the positive control group were sensitized with OVA/alum, received PBS as AIT and were challenged with nebulized OVA; mice in the negative control group were sensitized with PBS/alum, received PBS as AIT and were challenged with nebulized OVA; mice in the OVA-Therapy group were sensitized with OVA/alum, received OVA as AIT and were challenged with nebulized OVA; mice in the OVA-therapy + IL-4 Mutein group were sensitized with OVA/alum, received OVA as AIT combined with IL-4 Mutein and were challenged with nebulized OVA.

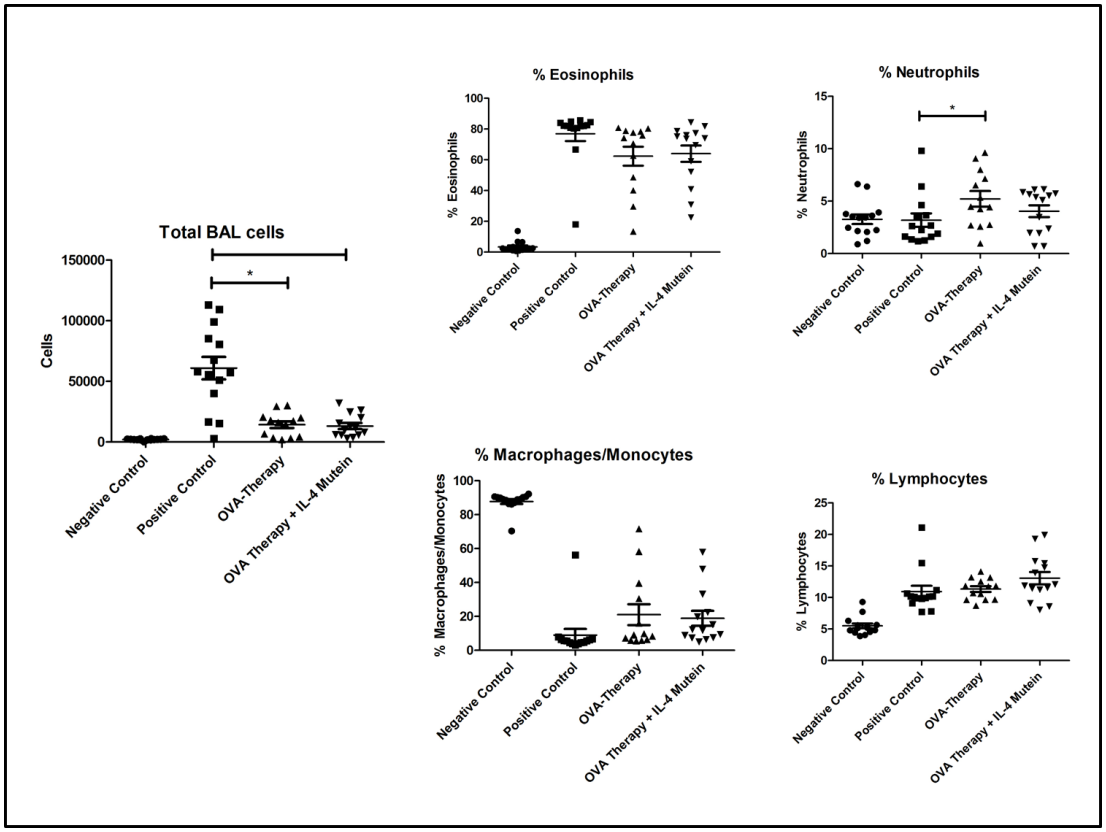

Figure 7.12: Effect of IL-4 Mutein treatment on BAL cells in the murine model "Allergen-specific immunotherapy" - Data are given as means +/- SEM (n=15). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

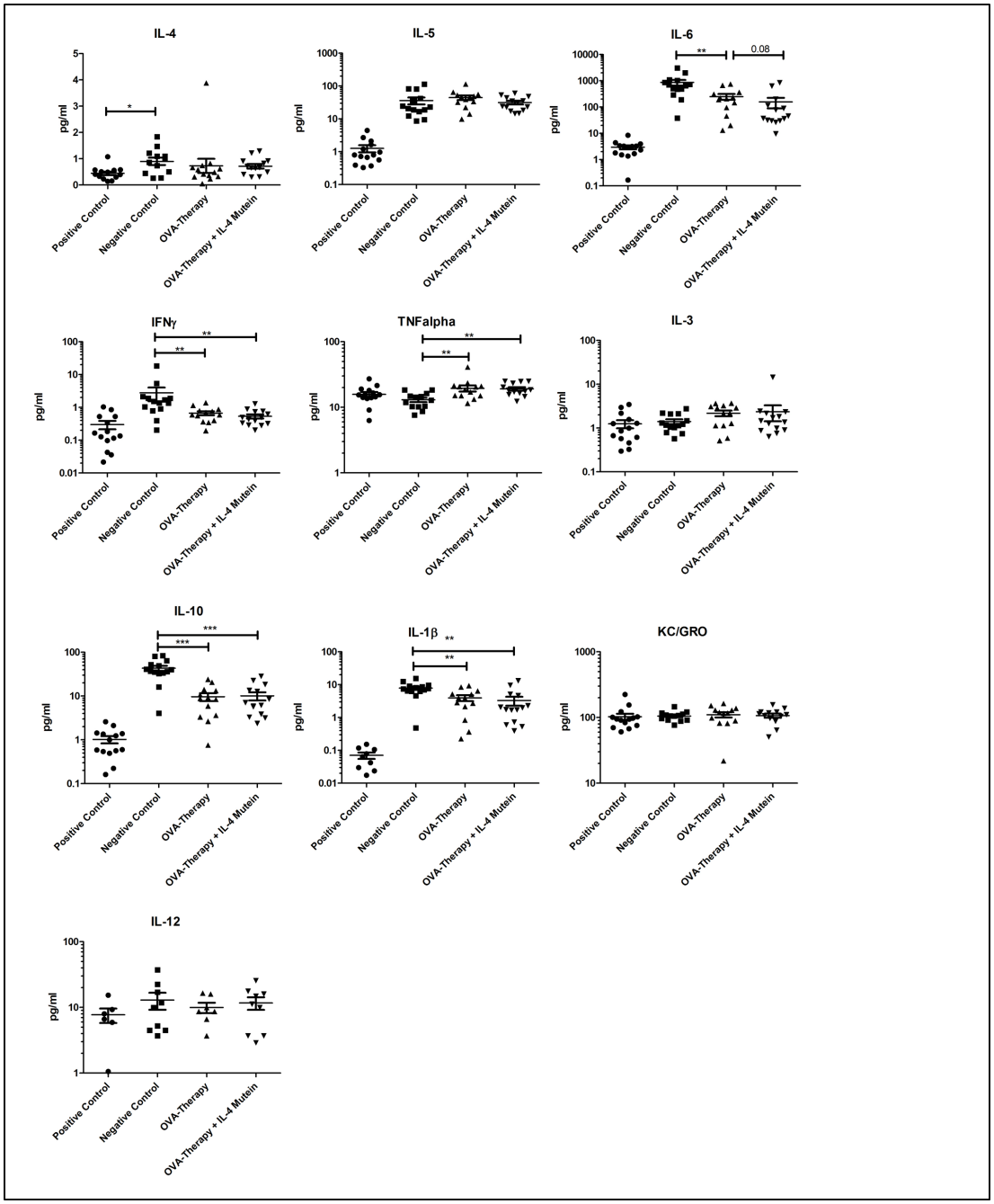

Figure 7.13: Effect of IL-4 Mutein treatment on BAL cytokine levels in the murine model "Allergen-specific immunotherapy" - Data are given as means +/- SEM (n=15). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

Further, cytokines in the serum were analyzed. Again, the sensitized and challenged mice in the positive control group were characterized by a cytokine pattern that strongly differed from the non-sensitized and challenged mice in the negative control group. IL-4, IL-5, IL-6 and IL-12p70 showed significantly higher concentrations in the positive control group. The OVA-therapy only had a minor effect on cytokine concentrations in the serum. Solely reductions in the levels of TNFα and IL-1β could be observed. The combined therapy of AIT and IL-4 Mutein did not only reduce the concentrations of TNFα and IL-1β but also led to a reduction of IL-5 in the serum. Due to the fact that IL-12 could only be detected in a small number of mice, a statistical analysis of this cytokine was not possible (Figure 7.14).

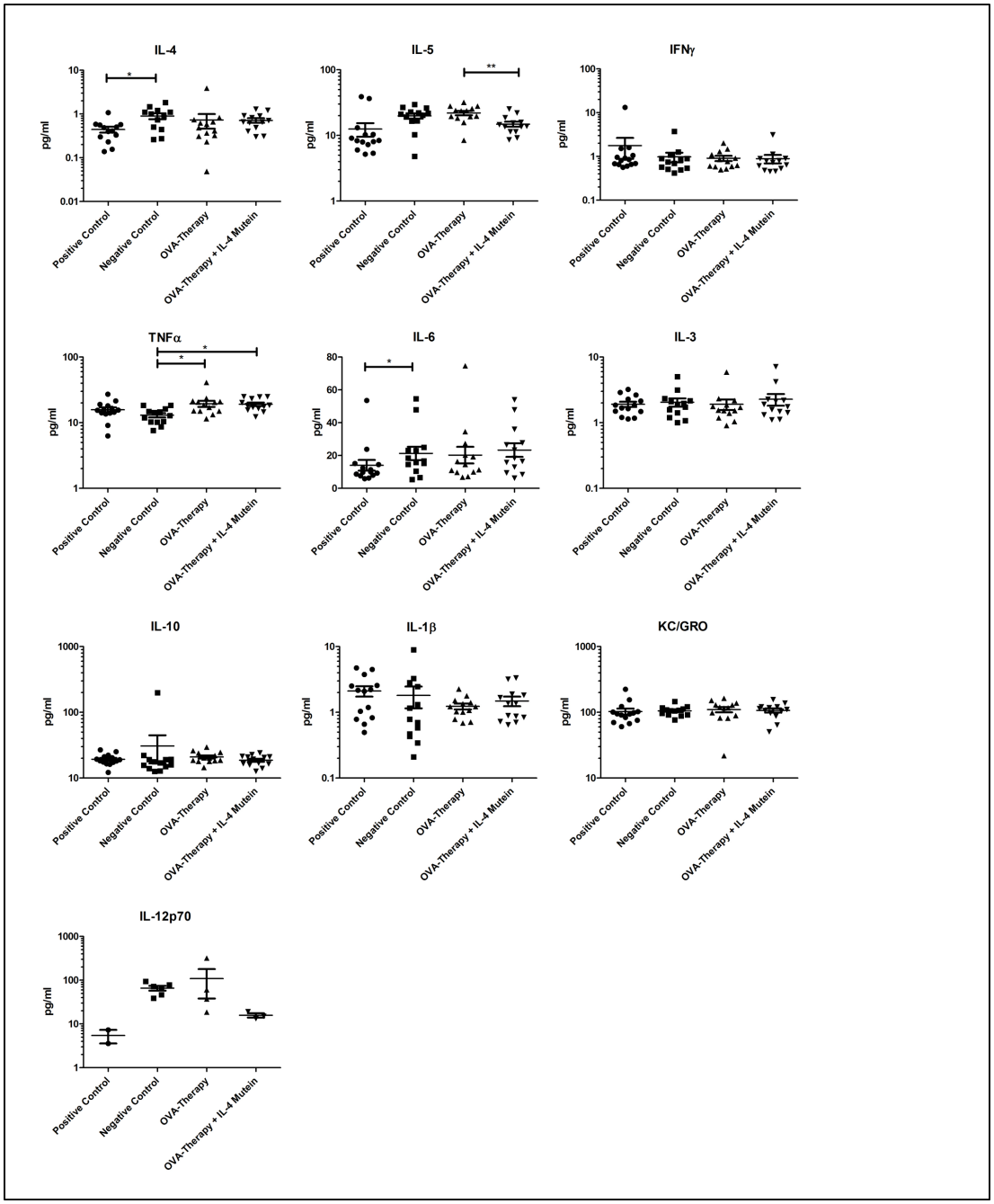

Figure 7.14: Effect of IL-4 Mutein on serum cytokine levels in the murine model "Allergen-specific immunotherapy" - Data are given as means +/- SEM (n=15). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

Next, the serum concentrations of several immunoglobulins were examined. Mice from the positive control group showed significantly higher levels of total IgE and OVA-specific IgE than mice from the negative control group. Also immunogobulins of the isotypes IgG1 and IgM showed a slight increase. Mice that received AIT were characterized by a significant reduction of total and OVA-specific IgE levels. Further, these mice were characterized by increased levels of IgG1 and IgA. The additive IL-4 Mutein treatment caused an almost significant increase of IgA antibodies. The remaining measured immunoglobulins were not affected by IL-4 Mutein (Figure 7.15).

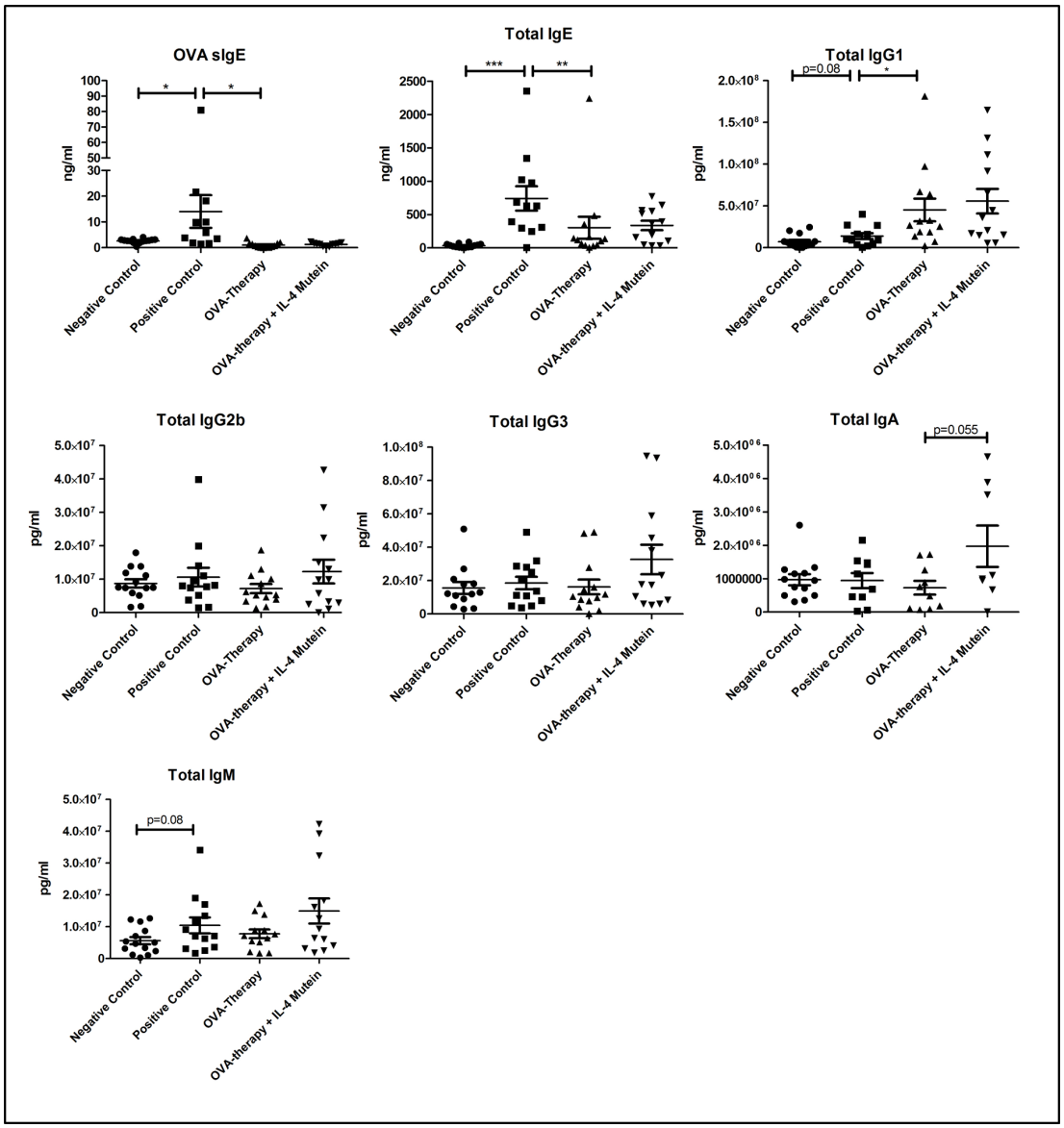

Figure 7.15: Analysis of serum-immunoglobulin levels after AIT in combination with IL-4 Mutein therapy - Data are given as means +/- SEM (n=15). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.6 Effect of IL-4 Mutein on the Efficacy of AIT during Chronic Allergen Exposure

7.6.1 Allergen Exposure Increases the Therapeutic Targets of IL-4 Mutein

A murine model of OVA-mediated lung inflammation was carried out to monitor the therapeutic targets of IL-4 Mutein before and after OVA-challenge (Figure 7.16). In accordance with the literature mice that were sensitized with OVA/alum and subsequently challenged with nebulized OVA showed increased concentrations of IL-4 and IL-13 in the BAL. Further, the levels of total IgE and OVA-specific IgE were increased compared with mice that were sensitized but not challenged (Figure 7.17).

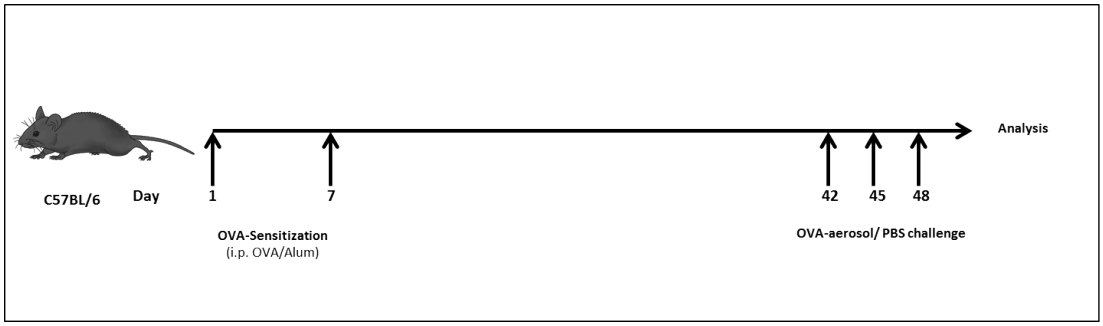

Figure 7.16: Murine model "Therapeutic targets of IL-4 Mutein" - Mice (C57BL/6J, n=15) were treated as follows: Mice from the challenged group were sensitized with OVA/alum before a subsequent OVA-aerosol challenge was performed; mice from the unchallenged group were sensitized with PBS/alum before the OVA-aerosol challenge was performed.

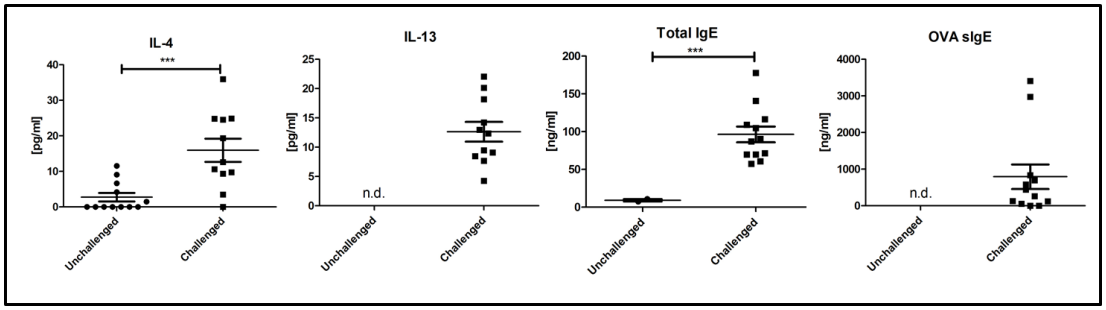

Figure 7.17: Therapeutic targets of IL-4 Mutein are increased after allergen challenge - Data are given as means +/- SEM (n=10). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.6.2 Short Term Effect of IL-4 Mutein and AIT on Allergic Lung Inflammation after Repetitive Allergen Exposure

In order to evaluate the short-term effect of AIT combined with IL-4 Mutein in a murine model with elevated levels of the therapeutic targets of IL-4 Mutein, OVA-sensitized mice were challenged during the phase of AIT (Figure 7.18). Alterations regarding the short-term cellular and humoral immune responses were analyzed one day after the last OVA-injection.

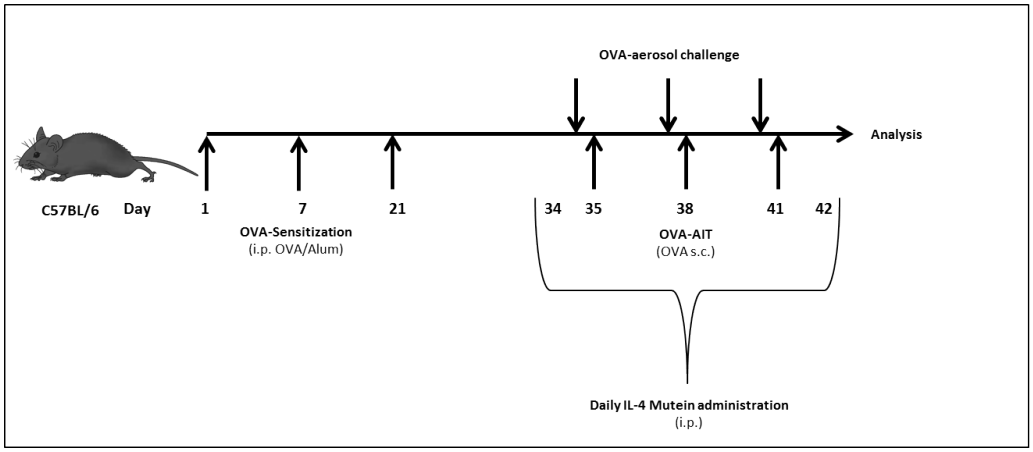

Figure 7.18: Murine model "Short term effect of IL-4 Mutein and AIT on allergic lung inflammation after repetitive allergen exposure" - Mice (C57BL/6J, n=10) were treated as follows: Mice in the positive control group were sensitized with OVA/alum before a PBS-AIT was performed during repetitive allergen challenges; mice from the negative control group weres sensitized with PBS/alum and received an PBS-AIT during repetitive OVA-challenges; mice in the OVA-Therapy group were sensitized with OVA/Alum and receivend an OVA-AIT during repetitiven OVA-challenges; mice in the OVA-Therapy +IL-4 Mutein group were sensitized with OVA/alum and received an OVA-AIT combined with IL-4 Mutein administration during repetitive OVA-challenges.

7.6 Effect of IL-4 Mutein on the Efficacy of AIT during Chronic Allergen Exposure

Mice from the negative control group that were sensitized with PBS/alum and received PBS AIT during repetitive challenges with nebulized OVA, showed no detectable OVA-specific IgE and only minor levels of total IgE. Mice from the positive control group, that were sensitized with OVA/alum and received PBS AIT during repetitive OVA challenges exhibited by high total IgE and OVA-specific IgE titers. Mice from OVA-Therapy group that were sensitized with OVA/alum and received OVA-AIT during OVA-challenges were characterized by an even higher serum concentration of total IgE. Additionally, they showed a strongly reduced abundance of OVA-specific IgE antibodies. Additional application of IL-4 Mutein during AIT in the OVA-Therapy + IL-4 Mutein group prevented the AIT-induced rise of the total IgE concentration. The abundance of OVA-specific IgE was even more decreased than after AIT alone. The level of OVA-specific IgG1 was strongly increased in the positive control group, the OVA-Therapy group and the OVA-Therapy + IL-4 Mutein group when compared to negative control group. However, the highest titers were observed in mice that underwent AIT alone. Significant levels of IgG2b antibodies were only found in mice that had received IL-4 Mutein (Figure 7.19).

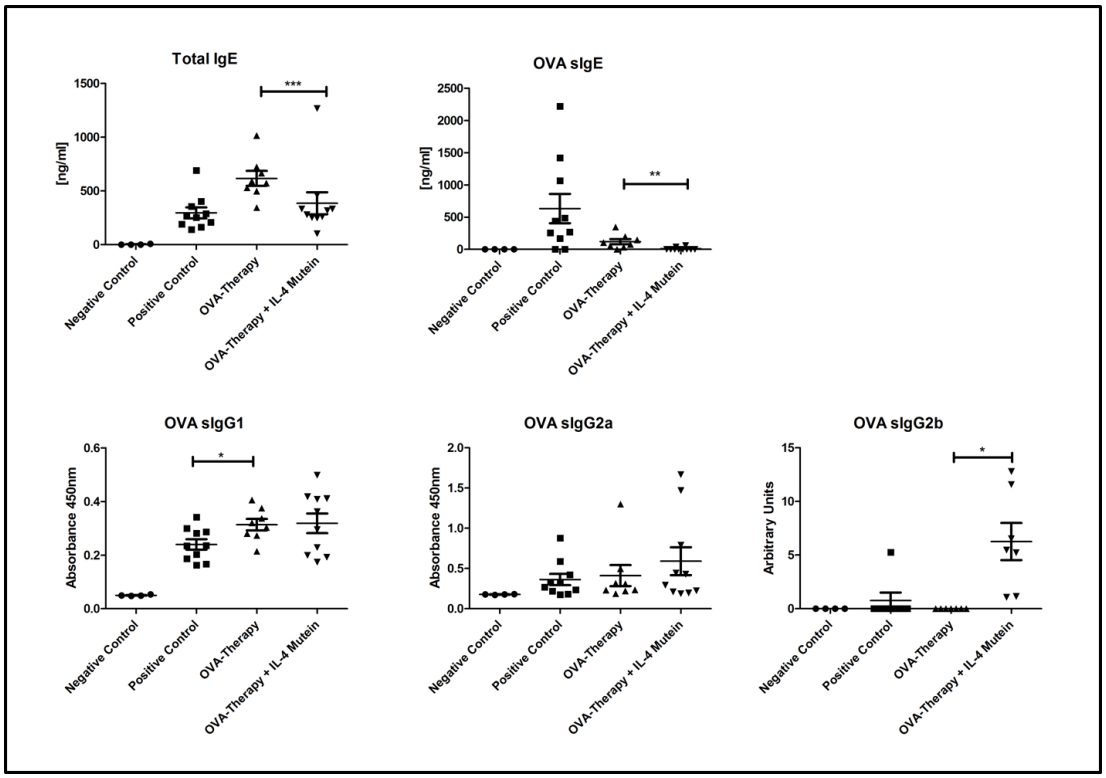

Figure 7.19: Impact of IL-4 Mutein on serum immunoglobulin levels in the murine model "Short term effect of IL-4 Mutein and AIT on allergic lung inflammation after repetitive allergen exposure" - Data are given as means +/- SEM (n=10). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.6 Effect of IL-4 Mutein on the Efficacy of AIT during Chronic Allergen Exposure

Significant levels of IL-4 Mutein were detectable in the BAL fluid via IL-4 ELISA 24 hours after the last IL-4 Mutein treatment. Mice from the positive control showed higher levels of IL-4 than mice from the AIT group. IL-5 concentrations were similar in all three groups. IL-13 was found in higher concentrations in the positive control than in the treated groups. IFNγ appeared to be present in lower amounts after AIT. Due to a strong mean variation in the IL-4 Mutein group, no statement can be made regarding IFNγ concentrations concerning the respective treatment. However, the levels of IL-6 were significantly decreased in both treated groups. Further, AIT appeared to induce higher levels of IL-17A that were suppressed by IL-4 Mutein administration (Figure 7.20).

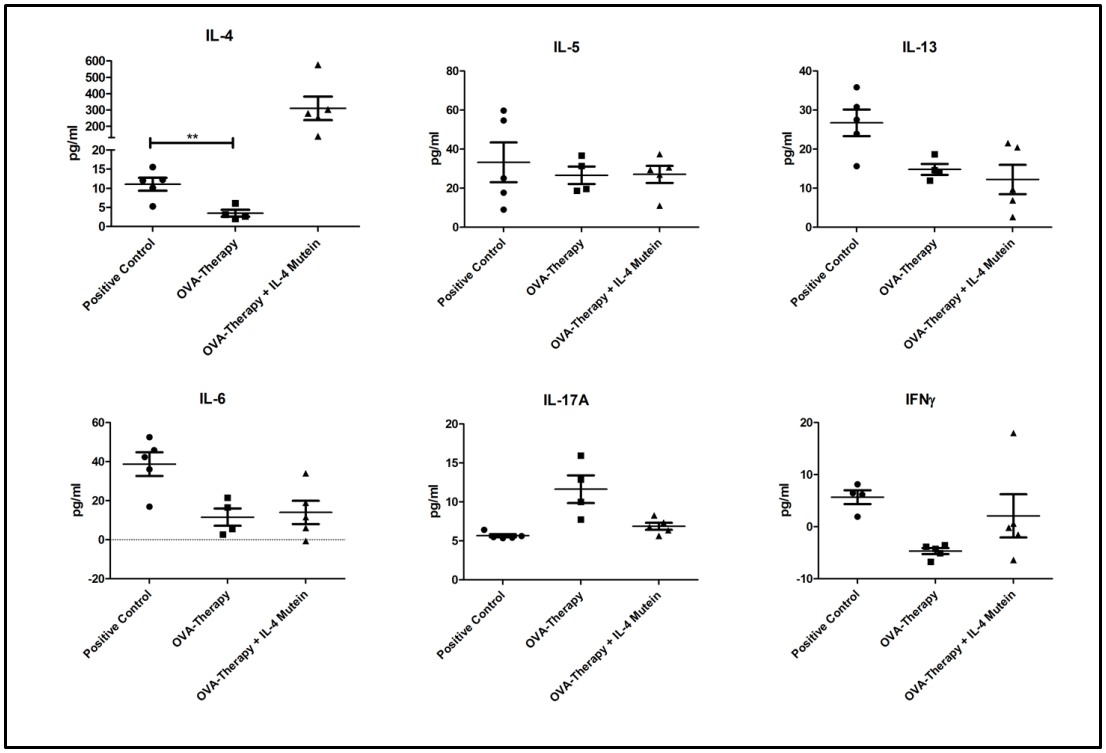

Figure 7.20: Impact of IL-4 Mutein on BAL cytokine levels in the murine model "Short term effect of IL-4 Mutein and AIT on allergic lung inflammation after repetitive allergen exposure" - Data are given as means +/- SEM (n=5). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

To evaluate the systemic effect of IL-4 Mutein combined with AIT, splenocytes of differently treated mice were isolated and restimulated with increasing doses of OVA. AIT did not have a significant impact on IL-4 production of restimulated splenocytes when compared to the positive control group. Splenocytes from IL-4 Mutein treated mice, however, showed almost no production of IL-4. Production of IL-13 was not decreased after AIT. In contrast, cells that were isolated from AIT and IL-4 Mutein mice showed significantly lower levels of IL-13 secretion. The same observation could be made for the production of IL-10. AIT alone only had a major impact on IL-5 levels in the supernatant. This significant reduction was not further affected by IL-4 Mutein treatment (Figure 7.21).

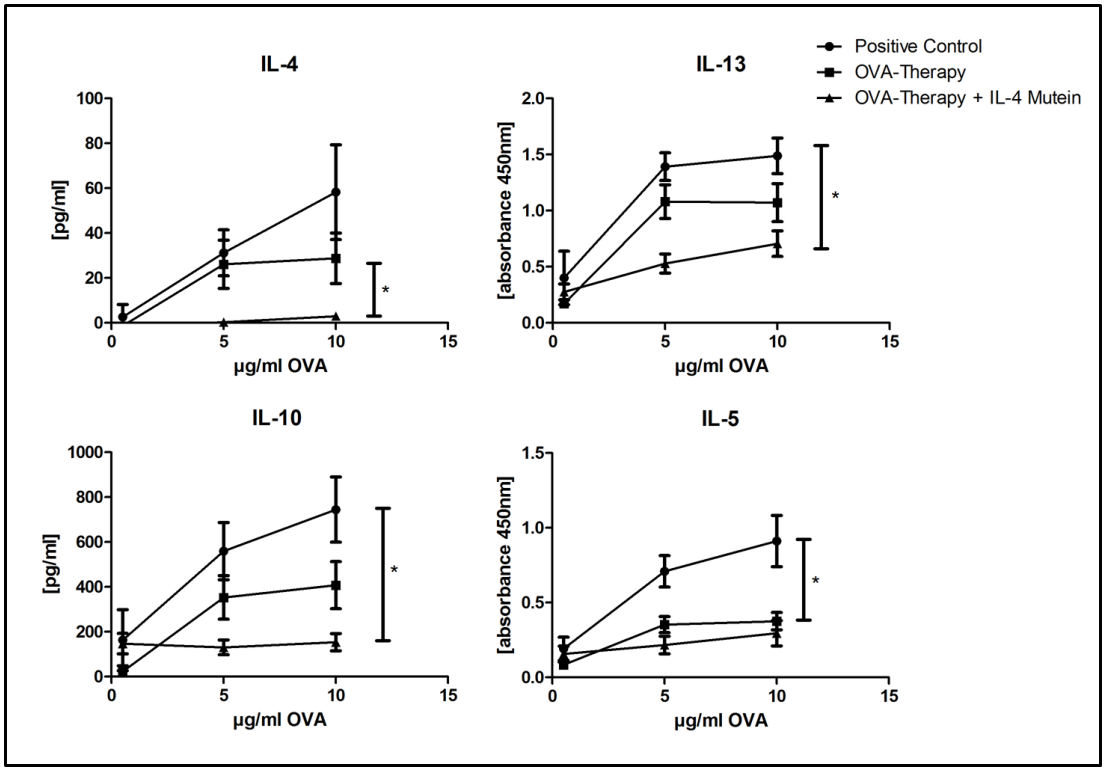

Figure 7.21: Analysis of cytokine secretion after antigen-specific restimulation of splenocytes - Data are given as means +/- SEM (n=5). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

As a tool to evaluate the effect of AIT combined with IL-4 Mutein on the clinical symptoms of lung inflammation, the BAL cells of treated mice were analyzed directly after the last therapeutic injection. Neither AIT alone nor the combination of AIT with IL-4 Mutein had significant impacts on the BAL cell numbers directly after therapy. Absolute cell numbers, total eosinophils, neutrophils, $CD4^+$ and $CD8^+$ cells were not altered in the mice that had undergone either therapy (Figure 7.22).

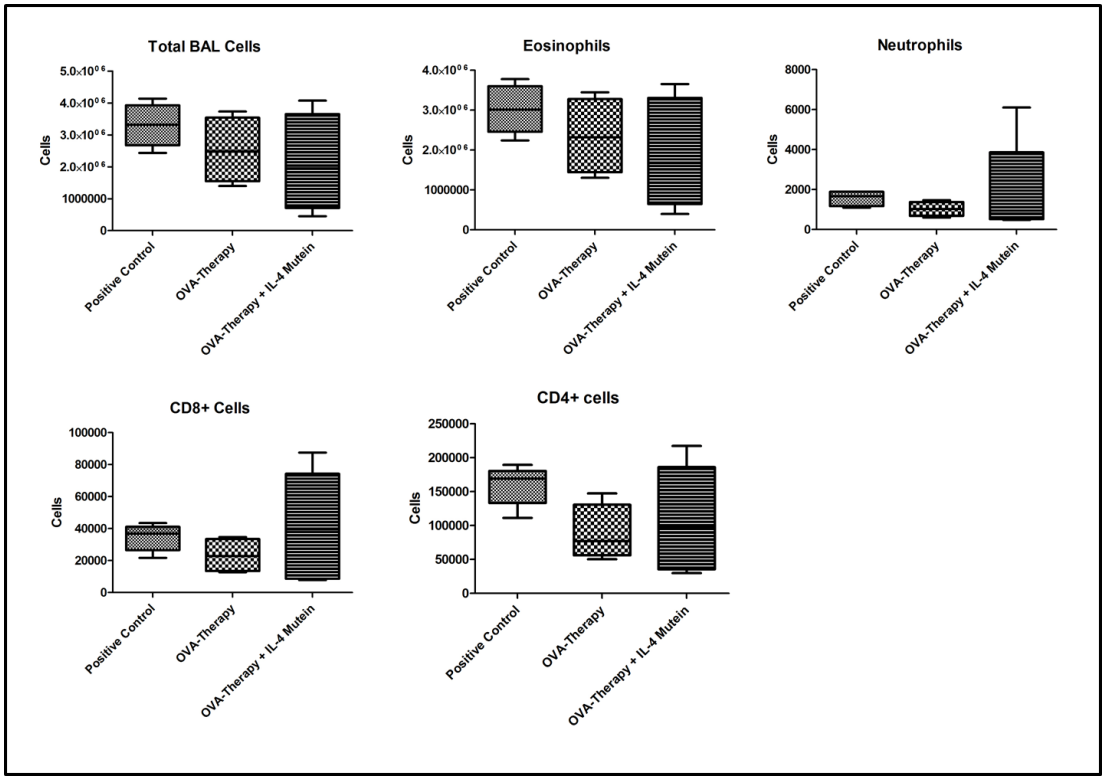

Figure 7.22: Analysis of BAL cells after AIT in presence or absence of IL-4 Mutein during repetitive allergen exposure - Data are given as means +/- SEM (n=5). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

In order to have a more detailed look at the effect of IL-4 Mutein on helper T cells, lymphocytes of differently treated mice were isolated from lung tissue and analyzed by flow cytometry. Mice from the group that received AIT in combination with IL-4 Mutein showed a significantly lower frequency of FoxP3-positive cells compared to mice from the positive control group. Amongst this subset, the frequency of the IL-33 receptor ST2 was decreased. The level of $ST2^+$ $FoxP3^+$ cells was significantly lower in mice that had received AIT in combination with IL-4 Mutein than in mice that had undergone AIT alone. The frequency of $FoxP3^+$ $GATA3^+$ cells was not significantly altered in the mice that had undergone AIT or AIT in combination with IL-4 Mutein when compared to positive control mice. Also the percentage of TH2 cells was similar in all three groups. AIT alone and the combination of AIT with IL-4 Mutein equally reduced the ratio of RorγT positive cells in the $CD4^+$ subset (Figure 7.23).

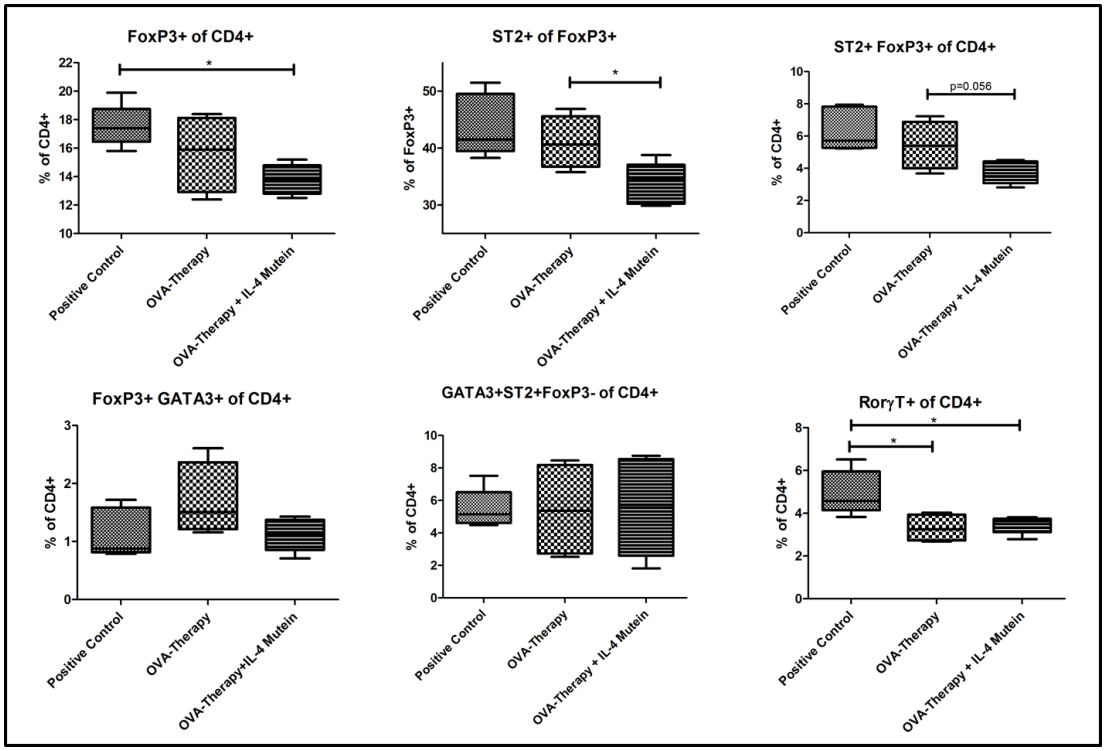

Figure 7.23: Analysis of lymphocytes after AIT in presence or absence of IL-4 Mutein during repetitive allergen exposure - Data are given as means +/- SEM (n=5). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.6.3 Long Term Effect of IL-4 Mutein Administration during AIT on Acute Lung Inflammation

In order to evaluate the long term effect of the combination of AIT with IL-4 Mutein on the immunological memory, mice that had undergone therapy during allergen exposure were challenged two weeks after their last therapeutic injection (Figure 7.24).

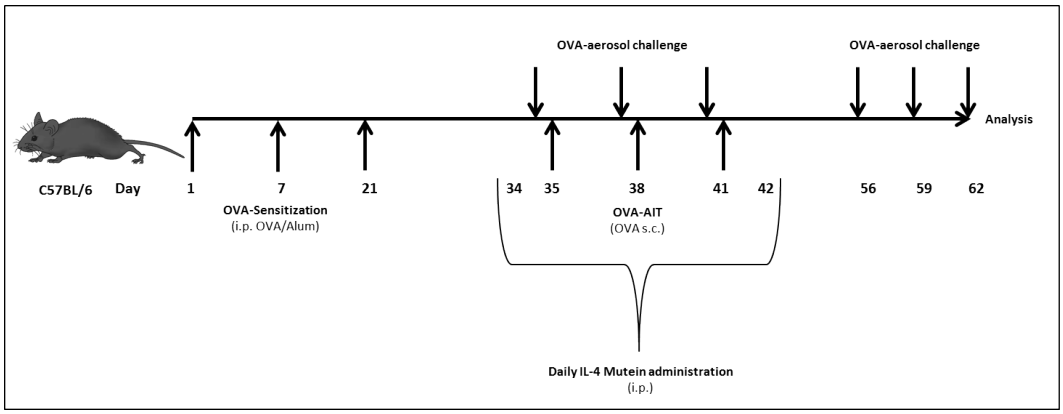

Figure 7.24: Murine model "Long term effect of IL-4 Mutein during AIT and repetitive allergen exposure" - Mice (C57BL/6J, n=10) were treated as follows: Mice in the positive control group were sensitized with OVA/alum before a PBS-AIT was performed during repetitive allergen challenges; mice from the negative control group were sensitized with PBS/alum and received an PBS-AIT during repetitive OVA-challenges; mice in the OVA-Therapy group were sensitized with OVA/alum and received an OVA-AIT during repetitive OVA-challenges; mice in the IL-4 Mutein Therapy group were sensitized with OVA/alum and received IL-4 Mutein administration during repetitive OVA-challenges Mice in the OVA-Therapy +IL-4 Mutein group were sensitized with OVA/alum and received an OVA-AIT combined with IL-4 Mutein administration during repetitive OVA-challenges.

Comparable to the results from the immunoglobulin measurement directly after the therapy (Figure 7.16), the total and OVA-specific IgE levels were increased in mice from the positive control group. These mice were sensitized wit OVA/alum and underwent PBS-AIT during challenges with nebulized OVA. Two weeks after the last AIT injection, mice were challenged again with nebulized OVA. Also OVA-specific IgG1 was increased in the positive control compared to the negative control. Mice from the negative control group were sensitized with PBS/alum and underwent PBS-AIT during repetitive challenges with nebulized OVA. Two weeks after the last AIT-injection, mice were challenged again with nebulized OVA. The negative control mice only showed a

detectable level of OVA-specific IgG2b. AIT did not have a significant impact on total IgE, OVA-specific IgE, IgG1 or IgG2b. In order to evaluate the long term effect of sustained IL-4 Mutein adminstration alone, a treatment group was introduced that did not receive OVA-specific AIT but IL-4 Mutein treatment alone. Mice from this IL-4 Mutein group did not show altered abundances of the measured immunoglobulins in the serum. The combined therapy of AIT and IL-4 Mutein was characterized by increased levels of OVA-specific IgG1 when compared to the positive control. The other measured immunoglobulins were not altered in this group (Figure 7.25).

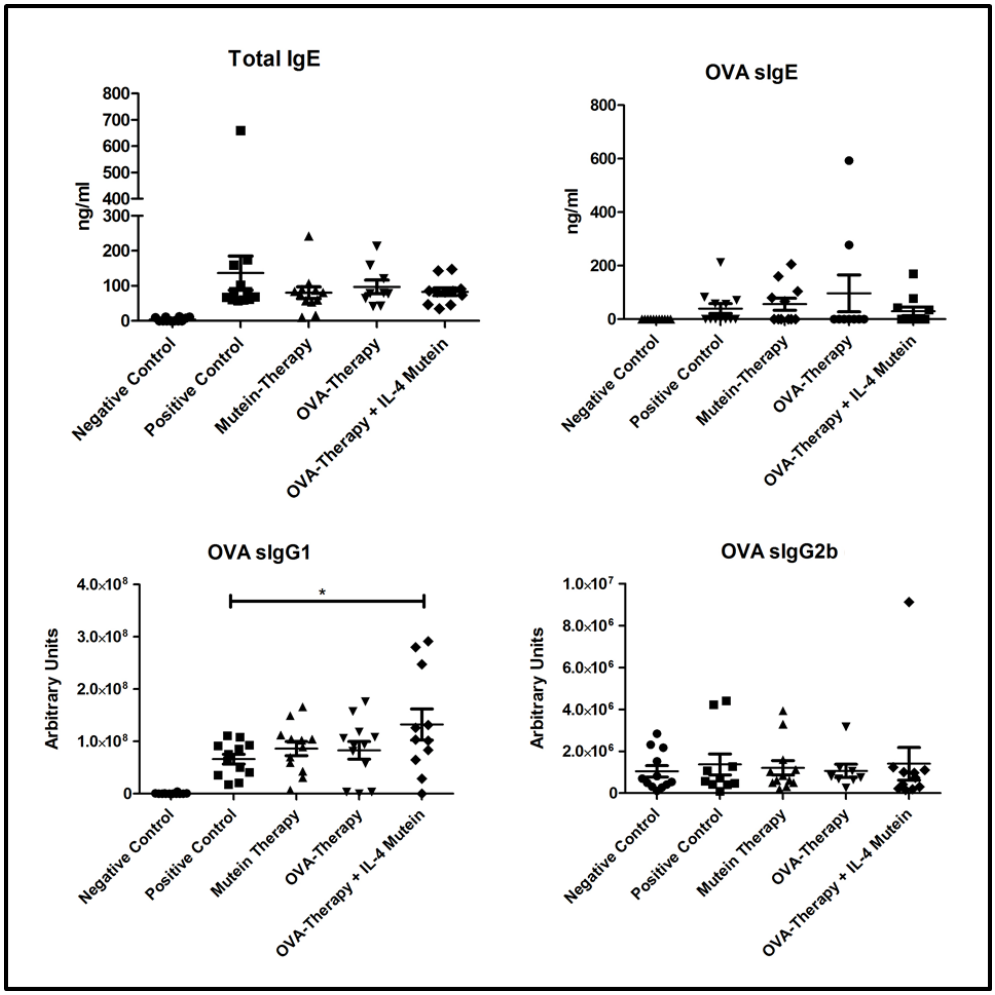

Figure 7.25: Impact of IL-4 Mutein treatment on the serum immunoglobulin levels in the murine model "Long term effect of IL-4 Mutein during AIT and repetitive allergen exposure" - Data are given as means +/- SEM (n=11). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.6 Effect of IL-4 Mutein on the Efficacy of AIT during Chronic Allergen Exposure

Next, cytokines in the BAL were measured via ELISA. IL-4 levels in the BAL of mice from the positive control group were strongly increased when compared to the negative control group. The rise could be significantly decreased by IL-4 Mutein treatment and AIT alone or the combined treatment of IL-4 Mutein and AIT. The abundance of IL-5 did not show a similar pattern. Untreated positive control mice showed comparable levels of IL-5 to mice from the negative control group. AIT alone significantly increased the concentration of IL-5. This elevation was not similarly pronounced in mice that had received AIT together with IL-4 Mutein or IL-4 Mutein alone. The level of IL-13 was strongly increased in the positive control group. AIT (in presence or absence of IL-4 Mutein) was able to reduce the IL-13 concentration. Mutein alone did not have an effect on the level of IL-13. The cytokine IL-17A was present in BAL samples of all groups in comparable amounts (Figure 7.26).

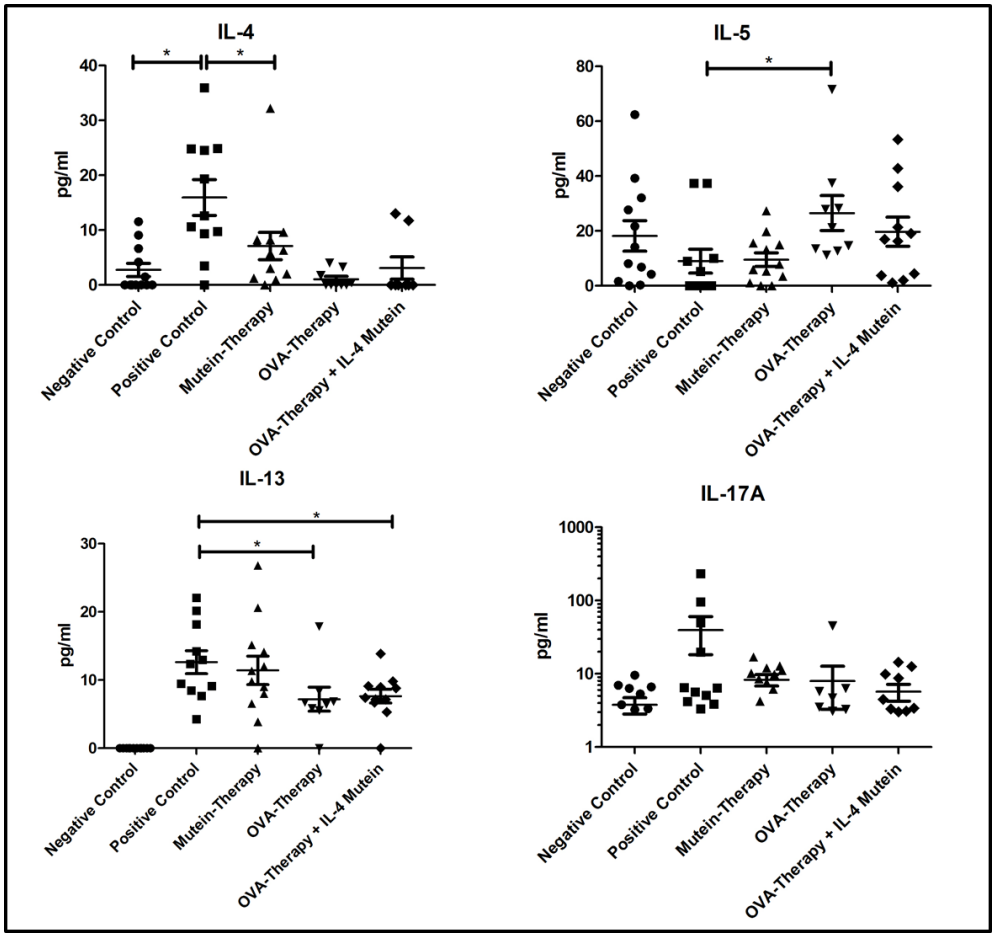

Figure 7.26: Impact of IL-4 Mutein treatment on the BAL cytokine levels in the murine model "Long term effect of IL-4 Mutein during AIT and repetitive allergen exposure" - Data are given as means +/- SEM (n=11). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.6 Effect of IL-4 Mutein on the Efficacy of AIT during Chronic Allergen Exposure

As a next step, infiltrated BAL cells were analyzed via FACS. Similar to the observations that were made directly after the therapy (Figure 3.19), the amount of total BAL cells was strongly increased in the positive control group. In this group, all measured subsets of leukocytes were increased when compared to the negative control group. AIT alone and IL-4 Mutein-supplemented AIT was able to reduce the total cell number in the BAL. The total amount of eosinophils, $CD4^+$ and $CD8^+$ cells was similarly decreased after AIT and AIT in presence of IL-4 Mutein. A further reduction of cell numbers via addition of IL-4 Mutein to AIT could not be observed. Strikingly, IL-4 Mutein treatment alone was capable of significantly reducing the number of total BAL cells when compared to the positive control. However, this effect was not as pronounced as in the groups that received OVA-AIT alone or in combination with IL-4 Mutein. The single cell subsets were not altered in the group that received IL-4 Mutein alone when compared to the positive control (Figure 7.27).

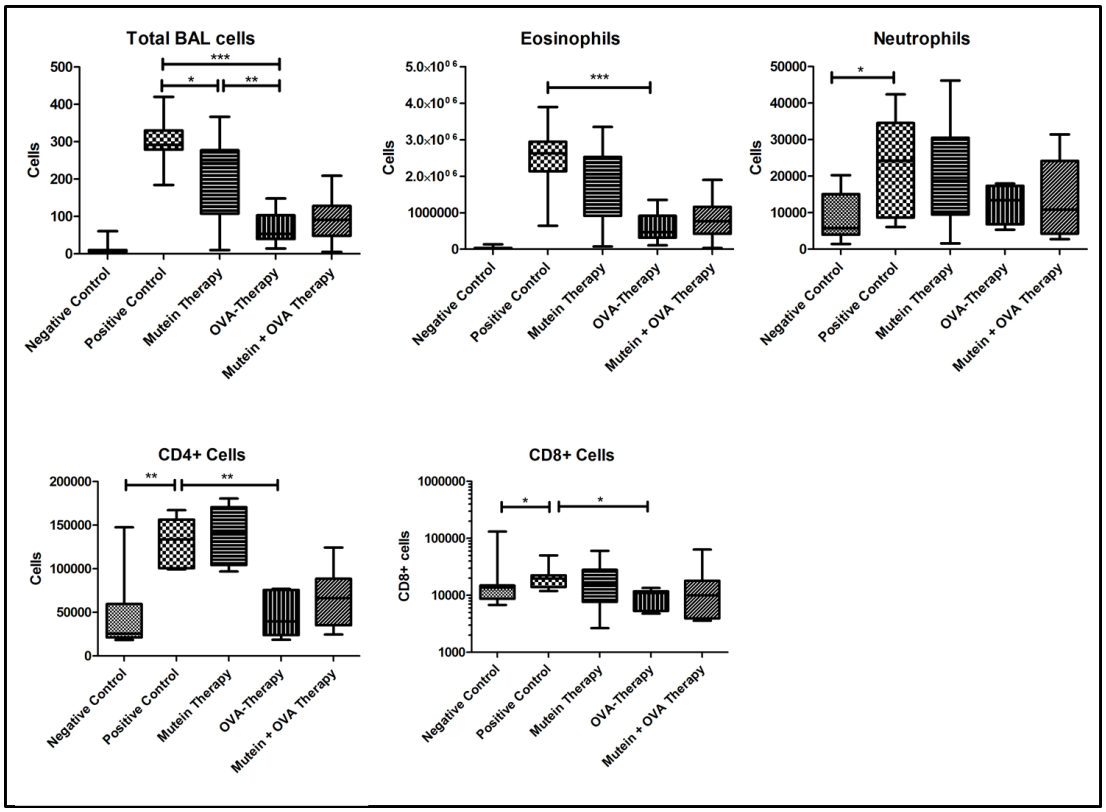

Figure 7.27: Analysis of BAL cells after combined AIT and IL-4 Mutein treatment after repetitive allergen exposure - Data are given as means +/- SEM (n=11). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.6 Effect of IL-4 Mutein on the Efficacy of AIT during Chronic Allergen Exposure

Lymphocytes were isolated from lung tissue in order to analyze the different populations of T helper cells of differently treated mice. The amount of $CD4^+$ T cells amongst $CD45^+$ cells was similar in all analyzed groups. The percentage of $FoxP3^+$ cells in the $CD4^+$ subset was strongly increased in mice from the positive control group. This increase was only reversed in the mice that received AIT in combination with IL-4 Mutein. Additionally, the fraction of $ST2^+$ cells among $CD4^+$ cells was highly elevated in mice from the positive control group when compared to the negative control group. This rise, however, could be suppressed by both, AIT alone and IL-4 Mutein spiked AIT. Interestingly, the majority of $ST2^+$ $CD4^+$ cells was $FoxP3^+$ in healthy mice from the negative control group. "Allergic" mice from the positive control group showed significantly less FoxP3 expression in $ST2^+$ $CD4^+$ cells. AIT alone resulted in a significantly larger fraction of $FoxP3^+$ cells in the ST2 CD4 double positive population than AIT combined with IL-4 Mutein. ST2 expression in the subset of $CD4^+$ $FoxP3^+$ cells was increased in the positive control group. No treatment led to changes in this specific subset. Mice from the positive control group were also characterized by an increased frequency of$ST2^+$ $FoxP3^+$ double positive $CD4^+$ cells. OVA-therapy alone resulted in a not significant reduction of this population. AIT combined with IL-4 Mutein resulted in a significant reduction of this cell subset (Figure 7.28).

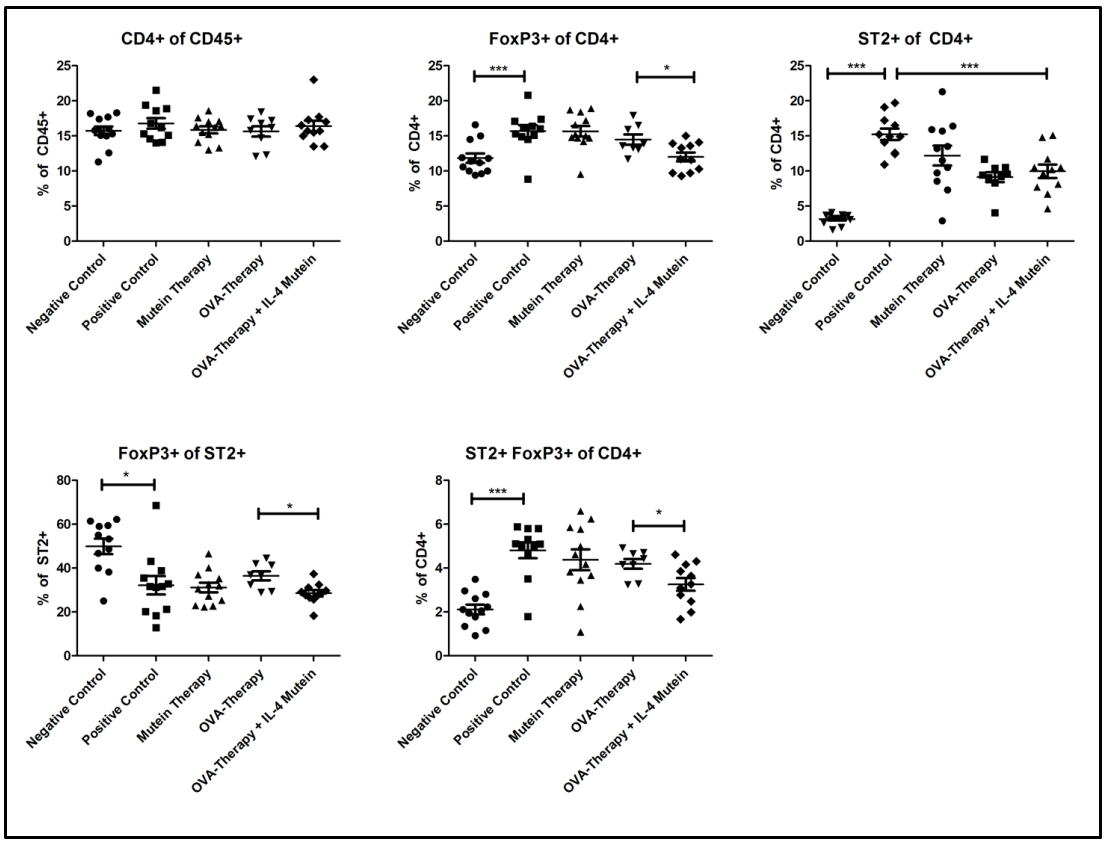

Figure 7.28: Analysis of lymphocytes from lung tissue after combined AIT and IL-4 Mutein treatment after repetitive allergen exposure I - Data are given as means +/- SEM (n=11). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

7.6 Effect of IL-4 Mutein on the Efficacy of AIT during Chronic Allergen Exposure

FoxP3 expression in GATA3$^+$ cells was significantly higher in mice from the negative control group than in all other treatment groups. Additionally, GATA3 expression in FoxP3$^+$ cells was strongly reduced in healthy negative control mice. Positive control mice showed an elevation of GATA3 expression in FoxP3$^+$ cells. This rise was not only reduced by the IL-4 Mutein-AIT combined therapy, but also by AIT or IL-4 Mutein alone. FoxP3$^+$ GATA3$^+$ double positive cells were twice as high in positive control animals than in mice from the negative control group. This increase was significantly reduced only by AIT under the umbrella of IL-4 Mutein. TH2 cells (CD4$^+$ GATA3$^+$ ST2 $^+$ FoxP3$^-$) cells were barely detectable in mice form the negative control group. In contrast, mice from the positive control group showed a much higher frequency of TH2 cells. AIT alone and in combination with IL-4 Mutein led to a significant decrease of TH2 cells among CD4$^+$ cells. Positive control mice showed higher expression of the transcription factor RorγT in CD4$^+$ T cells than negative control mice. AIT resulted in a decrease of RorγT$^+$ T cells. Combination with IL-4 Mutein led to a further significant reduction (Figure 7.29).

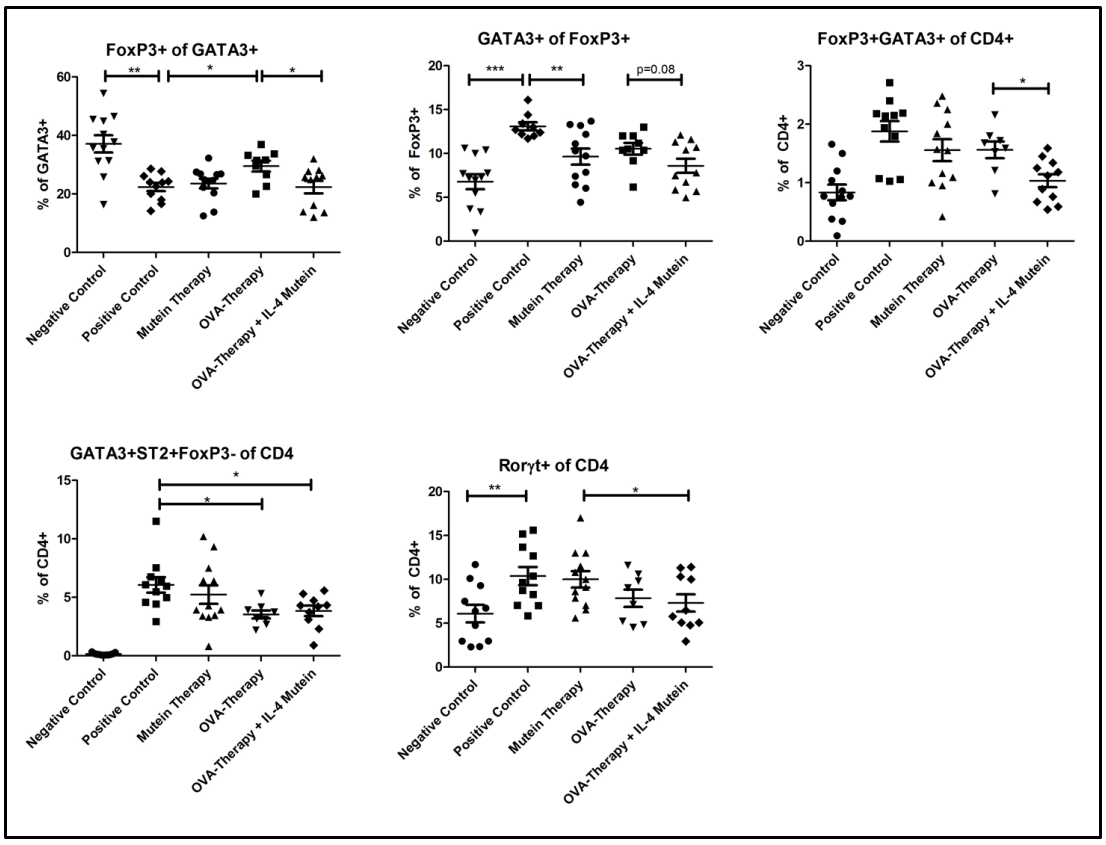

Figure 7.29: Analysis of lymphocytes from lung tissue after combined AIT and IL-4 Mutein treatment after repetitive allergen exposure II - Data are given as means +/- SEM (n=11). Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann Whitney test, respectively.

8

Discussion

The aim of this doctoral thesis was the therapeutic application of the IL-4 antagonist IL-4 Mutein in allergic diseases, especially as adjuvant during AIT. The first step of this project focused on the large scale production of IL-4 Mutein and the determination of its biologic activity. In contrast to all previous publications that analyzed the effect of IL-4 Mutein produced in either *Escherichia coli* (*E. coli*) or insect cells (Gogishvili et al., 2007), (Grunewald, Kunzmann, et al., 1997) and (Hahn et al., 2003), our approach focused on the expression and evaluation of IL-4 Mutein produced in mammalian cells. As the production in HEK293 and RAT-1 cells resulted in insufficient amounts for *in vivo* testing, in this thesis the challenging recombinant production of large amounts of IL-4 Mutein in CHO $DHFR^{-/-}$ cells using a DHFR carrying expression vector and the signal peptide of human serum albumin could finally be established. A yield of 8,5 mg/L was achieved after several rounds of methotrexate-mediated genomic amplification in serum-free medium. The resulting protein was demonstrated to be endotoxin-free using a limulus amebocyte lysate assay. Furthermore, the amount of correctly folded protein was much higher when compared to *E. coli*-derived IL-4 Mutein. This was demonstrated using an IL-4-specific ELISA, that only recognizes correctly folded IL-4 Mutein. Hence, IL-4 Mutein produced in mammalian cells proved to be superior in terms of biologic activity as well as in endotoxin contamination and, therefore, is more suitable for *in vivo* applications.

The functionality of IL-4 Mutein could be confirmed in various *in vitro* assays. The IC_{50} value of CHO-derived IL-4 Mutein was determined to a 25-fold excess over IL-4 Mutein to recombinant murine IL-4 using a cytotoxic T cell line whose survival is

dependent on IL-4 signaling. In concordance with the literature, IL-4 Mutein proved to be able to suppress IL-4-mediated differentiation of naive $CD4^+$ T cells into IL-4-producing TH2 cells. These results are conform with the results obtained by Grunewald, Kunzmann, et al., 1997 who originally developed murine IL-4 Mutein and tested it in CTLL-2 cells and other TH2-related *in vitro* settings. These and our observations underline the indispensable role of IL-4 for TH2 differentiation. However, in this project not only the effect of IL-4 Mutein on the differentiation of TH2 cells was analyzed, but also the role of IL-4 in the suppression of Treg induction. Although it is known that IL-4 suppresses TGFβ-induced FoxP3 expression (Mantel2007), in this work it was shown for the first time that IL-4 Mutein is able to rescue the IL-4-mediated suppression of FoxP3.

These two *in vitro* effects of IL-4 Mutein make it a very promising candidate for the supplementation of allergen-specific immunotherapy (AIT). Ideally, sustained inhibition of IL-4 receptor signaling could decrease the number of allergen-specific TH2 cells while supporting the induction of novel allergen-specific regulatory T cells during the course of AIT. Both IL-4 Mutein-mediated effects, the deletion of allergen-specific TH2 cells (Wambre et al., 2014) and the induction of Tregs (Radulovic et al., 2008), are known to be hallmarks of a successful traditional AIT. Hence, IL-4 Mutein might be an ideal adjuvant to enhance the efficacy of AIT. This might open new possibilities for the therapy of difficult to treat allergies or the treatment of patients who so far do not benefit from classical AIT such as polysensitized patients or patients with severe asthma.

In order to prolong the presence of IL-4 Mutein, which is characterized by a very short serum half-life, a PLGA-PEG-PLGA based hydrogel was tested as delivery matrix for its sustained release *in vivo*. The biodegradable (Choi et al., 2004), non-immunogenic (Cao et al., 2016) PLGA-PEG-PLGA triblock copolymer was synthesized and analyzed regarding its suitability as delivery matrix for IL-4 Mutein *in vivo*. The analysis of the chemical properties of polymer-PBS solutions revealed that polymer concentrations between 15% and 40% formed liquid solutions at room temperature and turned into a solid gel between 28 °C and 32 °C. Additionally, *in vitro* IL-4 Mutein release assays showed that the gelled polymer solution constantly releases stable amounts of IL-4 Mutein for at least 100 h. These characteristics make it an ideal candidate for the administration of proteins and other substances *in vivo*. In an *in vivo* release assay

it was demonstrated that 22 h after IL-4 Mutein injection, mice that had received IL-4 Mutein in a 20% hydrogel solution showed an IL-4 Mutein serum concentration twice as high than animals that had received IL-4 Mutein in PBS. This observation underlines the short serum half-life of IL-4 Mutein and makes PLGA-PEG-PLGA polymer solutions attractive delivery matrices for biologicals with short serum half-lifes.

Furthermore, the polymer was tested as delivery matrix of allergens during AIT. In a murine model of OVA-specific immunotherapy it could be shown for the first time that a PLGA-PEG-PLGA polymer solution represents a suitable delivery matrix for the the subcutaneous administration of allergens. Treatment of OVA-sensitized mice with high doses of OVA in PBS and OVA in polymer solution were similarly effective and resulted in a similar reduction of cell infiltration into the lung after allergen challenge. This ability indicates that the polymer represents a platform that could be loaded with allergens and diverse biologically active substances. A single injection with "loaded" hydrogel could enable a prolonged contemporaneous presence of allergen and immunomodulatory substances in the organism. Under consideration of the controversially discussed alum (Kramer and Heath, 2014), that is widely used as depot-forming substance for AIT, the development of novel matrices (e.g. microcrystalline tyrosine) (Leuthard et al., 2018) represents a promising and relevant research field.

The *in vivo* functionality of IL-4 Mutein could be shown in a model of alum-mediated sensitization to the model allergen OVA that mirrors the results from Grunewald, Werthmann, et al., 1998 who could demonstrate that prophylactic treatment with IL-4 Mutein can prevent an OVA-induced anaphylactic shock upon allergen challenge. In the model used in the this dissertation, preventive i.p. IL-4 Mutein treatment before and after i.p. alum sensitization to OVA resulted in a significantly altered OVA-specific and total immunoglobulin-profile: Besides lower levels of total IgE and OVA-specific IgE in IL-4 Mutein-treated mice, increased levels of total IgG1, IgG2a, IgG2b, IgG3 and IgM could be observed in the serum. Interestingly, IgG2a, IgG2b and IgG3 have previously been found to be strongly increased in IL-4-knock-out mice (Kuhn et al., 1991). The observed increase in IgG1 serum concentrations, however, is surprising. In similar *in vivo* settings the presence of IL-4 was necessary for the class switch to IgG1 (Fischer et al., 2002). However, disease induction via alum seems to be an exception to this rule. Brewer et al., 1996 and his colleagues observed a strong alum-mediated IgG1 induction in IL-4 deficient mice, a fact that was confirmed by the results of this work.

Preventive treatment with IL-4 Mutein also had some interesting effects on serum cytokine levels: First of all, IL-4 Mutein was detectable in large concentrations using the IL-4 detection antibodies. Whereas a single sensitization with OVA complexed with alum resulted in the reduction of IL-1β, IL-6 and TNF-α, IL-4 Mutein treatment reversed these reductions so that these cytokines could be found in identical amounts in non-sensitized and IL-4 Mutein treated mice. Furthermore, the TH2 key cytokine IL-5 was decreased in the serum. A possible explanation for this reduction could be found in a decreased number of TH2 cells. However, as this murine model did not include an allergen-challenge after the sensitization only minor systemic changes were expected.

Taken together, the preventive treatment of mice with IL-4 Mutein demonstrated that CHO-derived IL-4 Mutein is functional *in vivo*. The same sensitization-preventing effect could be shown when IL-4 Mutein was administered not i.p. in PBS but s.c. in a 20% PLGA-PEG-PLGA in PBS solution (data not shown).

In the model of house dust mite (HDM)-induced lung inflammation, the therapeutic effect of IL-4 Mutein treatment during the challenge phase with HDM-extract was analyzed. The significant decrease of BAL cells and total IgE after HDM-challenge confirms the results from Piyadasa et al., 2016 who identified IL-4 and IL-13 as central players in the murine model of HDM-induced allergic airway inflammation. Furthermore, other studies that used cytokine-blocking approaches could observe that blocking of IL-13 only prevented airway pathology caused by HDM-exposure (Tomlinson et al., 2010). The observed strong therapeutic effect that could be observed in this work proves that i.p. injections of 100 µg IL-4 Mutein are sufficient to suppress IL-4 receptor-mediated signaling even in the powerful model of HDM-induced lung inflammation.

Whereas the previous experiments only aimed at characterizing IL-4 Mutein and PLGA-PEG-PLGA polymer solutions, the actual aim of this project was to test the adjuvant effect of sustained suppression of IL-4 receptor signaling during AIT. This experimental setup resembles the experiments carried out by Gogishvili et al., 2007 who also aimed at improving AIT via blocking of IL-4 receptor signaling. However, different shortcomings of this publication that resulted in mainly negative results regarding IL-4 receptor blocking during AIT, should be overcome: Firstly, a CHO-derived IL-4 antagonist was used instead of a bacterial one. Further, under consideration of the short serum half-life of IL-4, a ten-fold higher dose was applied. Additionally, this dose

was not administered in PBS, but instead in a PLGA-PEG-PLGA-based hydrogel that prolongs the serum half-life of IL-4 Mutein, as demonstrated.

Unfortunately, also in the altered experimental setup in this work the combination of IL-4 Mutein with AIT did not result in a superior therapy outcome when compared to the traditional AIT alone. Neither absolute BAL cell numbers nor the ratio of different BAL compounds were affected by the supplemented therapy. Also cytokines in the BAL were similar in both treatment groups. Solely the levels of IL-6 in the BAL were slightly decreased in mice that had received AIT in combination with IL-4 Mutein. Immunoglobulin levels were barely affected. Only IgA levels were slightly increased in mice that had received AIT in combination with IL-4 Mutein. Moreover, the analyses of cytokine levels in the serum showed a significant reduction of the IL-5 level under combined treatment with AIT and IL-4 Mutein. Disappointingly, this systemic reduction of the TH2 key cytokine IL-5 that is important for many characteristics of allergic inflammation (Leckie et al., 2000) did not result in a less severe disease upon allergen exposure.

However, a closer look at the therapeutic targets of IL-4 Mutein (IL-4, IL-13 and IgE) in unchallenged mice demonstrated that only allergen-challenge of OVA-sensitized mice led to a marked increase of these serological TH2 markers that can also be found in atopic human patients (Hashimoto et al., 1993), (Tavakkol Afshari et al., 2007) and (Burrows et al., 1989). Due to this fact, a novel murine model of AIT that is characterized by repetitive allergen challenges and increased TH2 markers during AIT was established in this work.

In this new mouse model, two different time points were analyzed. The first time point aimed at elucidating the immediate effects of IL-4 Mutein supplemented AIT and was carried out shortly after a treatment-block consisting of repetitive allergen challenges, AIT injections and daily IL-4 Mutein administrations. The second endpoint was chosen to determine the long term effects of IL-4 Mutein supplemented AIT. For this time point mice were kept for two more weeks before another challenge block was carried out.

Interestingly, treatment with IL-4 Mutein in this model resulted in numerous changes on immunoglobulin levels at the first time point. As expected, total IgE and OVA-specific IgE levels were decreased in mice that had received IL-4 Mutein during AIT.

Furthermore, levels of the OVA-specific blocking antibody IgG2b were significantly increased after IL-4 Mutein treatment. These changes can be explained by the influence of IL-4 on isotype-switching. Whereas it is common knowledge that IL-4 induces the IgE class-switch, the inhibitory effect of IL-4 on TGF-β-induced IgG2b secretion is less known (Deenick et al., 2005). Furthermore, OVA-AIT alone induced a rise in total IgE and OVA-specific IgG1 that could not be observed in mice that had received IL-4 Mutein during AIT. In addition to these changes on immunoglobulin level, also the levels of cytokines in the BAL were altered. Whereas OVA-AIT alone induced a significant reduction of IL-4 in the BAL, the presence of IL-4 Mutein in the BAL made it impossible to quantify the body´s own IL-4 in the BAL of IL-4 Mutein treated mice. The TH2 cytokine IL-13 and the proinflammatory cytokine IL-6 were already strongly reduced by AIT alone. Administration of IL-4 Mutein did not result in a further reduction. Only IL-17A, a cytokine associated with severe asthma (Chesne et al., 2014) was increased after AIT alone. This increase could be completely abolished by IL-4 Mutein administration. The mechanism and meaning of this IL-4 mediated IL-17A suppression needs further investigation.

Surprisingly, despite of the decreased OVA-specific IgE levels which proof the functionality and sufficient dosing of IL-4 Mutein, the infiltration of immune cells into the lung was not significantly decreased after i.p. IL-4 Mutein treatment at time point one when compared to the traditional AIT treatment. These results are in contradiction with the results from Henderson et al., 2000 who observed a decreased airway inflammation following allergen challenge in an alum-mediated mouse model after i.p. injection of soluble IL-4 receptor α. Also Tomkinson et al., 2010 made similar observations using another IL-4 receptor antagonist. However, Hahn et al., 2003, who used the very same IL-4 Mutein that is used in this study, also could not observe significant changes on BAL cytokine levels, airway inflammation or eosinophilia. A reason for this deviation could be the shorter serum half-life of IL-4 Mutein or a comparatively lower presence of the respective biological in the lung. Another cause for this observation could be the simultaneous administration of high allergen doses during IL-4 Mutein treatment that could somehow interfere with the efficacy of IL-4 Mutein.

In contrast to the non-detectable effect in the lung, the administration of IL-4 Mutein induced several changes on a systemic level. Antigen-specific restimulation of splenocytes with different doses of OVA resulted in a significantly decreased production

of IL-4 by splenocytes from mice that had received AIT in presence of IL-4 Mutein. This reduction was not as prominent in splenocytes from mice that had received AIT alone. Other TH2-associated cytokines like IL-13, IL-10 and IL-5 were decreased by trend in splenocytes from mice that had received IL-4 Mutein during AIT when compared to mice that had received AIT alone. These results indicate that the sustained inhibition of IL-4 receptor signaling does indeed affect the number of TH2 cytokine-producing cells or their ability to secrete these cytokines without affecting ongoing local inflammation upon allergen challenge.

Whereas IL-4 Mutein supplementation did not result in significant changes on cells in the BAL, it had some interesting effects on T cell populations in the lung tissue. Strikingly, the fraction of FoxP3$^+$ cells among CD4$^+$ cells was decreased in IL-4 Mutein-treated mice. This finding is for several reasons surprising. Firstly, the *in vitro* experiments performed in this study resulted in the opposite effect of IL-4 Mutein as they showed that suppression of IL-4 receptor signaling is able to rescue FoxP3-expression. Moreover, numerous publications could associate the number of FoxP3$^+$ Tregs with the state of tolerance in mice (Bohm et al., 2015) and men (Kerstan et al., 2011). In this study the number of Tregs did not show a negative correlation to disease severity. As especially activated, ST2$^+$ FoxP3$^+$ cells were decreased among the CD4$^+$ subset in mice that were treated with IL-4 Mutein and AIT when compared to AIT-treated mice, one could assume that this regulatory cell population does not correlate with the state of tolerance. Recently, this population could even be shown to be characterized by a TH2-biased phenotype (Siede et al., 2016). If these cells turn out to be critically involved in the disease mechanism of allergic diseases, IL-4 receptor inhibition could represent a promising therapeutic approach to target these cells.

Yet another unexpected observation at time point one was the number of TH2 cells in the lung tissue. Whereas the differentiation of TH2 cells could be suppressed by IL-4 Mutein *in vitro*, the number of these GATA3$^+$ST2$^+$FoxP3$^-$ was not altered in mice that had received IL-4 Mutein during AIT when compared to mice that had received AIT alone. GATA3$^+$ Foxp3$^+$ cells, that are known for their "TH2-like phenotype" (C. C. Chen et al., 2017) did not show a significant decrease in mice that had received IL-4 Mutein in combination with AIT. Another unexpected observation was that AIT alone or in combination with IL-4 Mutein could significantly decrease the abundance of Rorγt$^+$ cells among the CD4$^+$ subset. This observation that was also made by other

groups (Shin et al., 2017) illustrates that AIT does not only affect TH2 and regulatory T cells but has various effects on different cell types.

However, as the first time point was only meant to give insights into the acute cellular and molecular changes during IL-4 Mutein-supplemented AIT. The main read-out was performed two weeks and one challenge block later in order to evaluate the influence of IL-4 Mutein on the long-term effect of AIT. At this time point, an additional test group was analyzed. In order to exclude effects of IL-4 Mutein administration alone and to highlight the combined effect of IL-4 Mutein and AIT, a group of mice was treated with IL-4 Mutein without receiving AIT.

At this time point the previously reduced total and OVA-specific IgE levels in the IL-4 Mutein- and AIT-treated mice had increased to the level of the positive control mice. The same observations could be made for the levels of OVA-specific blocking antibodies of the IgG2b subtype that were similar in all treated groups at time point two. Solely OVA-specific IgG1 was significantly increased in the IL-4 Mutein and AIT group when compared to all other groups.

Also the BAL cytokine levels, that had shown minor differences between the mice that had received AIT alone or AIT in combination with IL-4 Mutein at time point one, were not affected by the administration of IL-4 Mutein during AIT at time point two. All measured cytokines showed similar levels in the traditional and the IL-4 Mutein-supplemented AIT group. However, already the AIT alone led to strong decreases of IL-4 and IL-13 levels. Strikingly, IL-4 Mutein administration without AIT also led to a significant decrease of IL-4 levels. This indicates that the sustained inhibition of IL-4 receptor signaling alone could have an effect on IL-4 production. The exact cell type affected by this IL-4 deprivation, however, has yet to be identified.

The BAL cell numbers confirmed the results obtained for the BAL cytokines. AIT alone led to a strong decrease in total BAL cells, eosinophils, $CD4^+$ and $CD8^+$ cells. Of note, this effect of AIT on BAL cells was not significant at the earlier time point when compared to the positive control. Combination of AIT with IL-4 Mutein did not induce further improvements. The most unreckoned result regarding the BAL cell number was the effect of sustained IL-4 Mutein treatment alone which resulted in a significant decrease of total BAL cells. This further illustrates that a prolonged IL-4 receptor signaling suppression leads to lasting changes.

The decreasing effect of AIT on the number of FoxP3$^+$ cells that was observed at time point one could be confirmed at time point two. Mice from the positive control group showed significantly higher numbers of FoxP3$^+$ cells among the CD4$^+$ subset than healthy mice. Whereas OVA-AIT alone only led to a non-significant decrease, treatment with IL-4 Mutein during AIT showed a significant decrease when compared to mice from the positive control group. As mice from the positive control group were severely sicker than mice from the AIT-groups it is clear that the number of FoxP3$^+$ cells alone does not provide information about the immunological tolerance of an organism. Also studies in humans observed increased numbers of Tregs in asthmatic patients (Landgraf-Rauf et al., 2016). One could speculate whether rather the quality and not the quantity of regulatory T cells is decisive for tolerance induction. However, this topic still needs further investigation.

AIT alone also had a strong impact on the expression of the IL-33 receptor ST2. Whereas this receptor was barely expressed in mice from the negative control group, it was strongly upregulated in mice from the positive control group. This rise could be halved by AIT alone and AIT in combination with IL-4 Mutein. Although the addition of IL-4 Mutein had no additional effect, this strong impact of AIT is an interesting observation as the influence of AIT on the IL-33 receptor has not been described in the literature, yet. This effect could also be interesting for AIT in humans, as IL-33 is a well known inducer of TH2 cytokine production in various cell types (Savinko et al., 2013), a fact that makes it an attractive therapeutic target. However, not only the general number of ST$^+$ cells was affected in our model. Especially the ST$^+$ FoxP3$^+$ cells were affected. This sub-population of Tregs that has been associated with a TH2-like phenotype was strongly increased in mice from the positive control group. AIT alone did not result in a reduction of these cells. Only the combination of IL-4 Mutein with AIT could significantly reduce the number of ST$^+$ FoxP3$^+$ among CD4$^+$ cells. The link between IL-4 receptor signal inhibition and the local suppression of TH2-like Tregs has still to be identified.

Whereas in healthy mice from the negative control group, almost half of the GATA3$^+$ cells were FoxP3$^+$, their amount was barely half the size in mice from the positive control group. AIT alone led to a small but significant increase of FoxP3$^+$ expressing GATA3$^+$ cells that could not be observed in mice that had received IL-4 Mutein during AIT. This could imply that IL-4 Mutein treatment impairs the expression of FoxP3

in numerous CD4 sub populations. The other way around it was interesting to see that healthy mice from the negative control group showed a weak expression of GATA3 in FoxP3$^+$ cells that was doubled in mice from the positive control group. AIT and IL-4 Mutein therapy alone led to a significant decrease of GATA3 expression. The combined AIT with IL-4 Mutein showed an even lower fraction of GATA3+ cells in the FoxP3+ subset. The same observation could be made for FoxP3$^+$ GATA3$^+$ cells among CD4$^+$ cells. It is imaginable that these so called "ex-regulatory Tregs" that are associated with TH2 cytokine production in humans (Reubsaet et al., 2013) and mice (C. C. Chen et al., 2017) only develop in presence of IL-4 receptor signaling. IL-4 receptor inhibition could therefore lead to the suppression of differentiation of this cell type. Depending on the importance of this cell-subset for allergic diseases, IL-4 receptor inhibition could represent an interesting treatment option.

The so called pathogenic TH2 cells which are GATA3$^+$ ST2$^+$ FoxP3$^-$ were barely detectable in healthy mice and strongly increased in mice from the positive control group. IL-4 Mutein alone had no effect, whereas AIT in presence or absence of IL-4 Mutein showed an equal decrease of this widely studied cell type. This result goes in line with other studies that observed that IL-4 receptor signaling is decisive for TH2 differentiation but has no further effect on these cells as soon as TH2 priming has occurred (Corry et al., 1996).

The fraction of Rorγt$^+$ cells in the CD4$^+$ subset was significantly increased in all treatment groups at time point two when compared to time point one. This increase is most likely caused by the repetitive allergen exposure that leads to a prolonged lung inflammation in all mice and is characterized by a mixed TH2/TH17 immune response (Zhao et al., 2013). Consistently with the observations made at time point one, Rorγt expression was significantly reduced after AIT or AIT in combination with IL-4 Mutein treatment, underlining the long-term effect of AIT on various cell types apart from TH2 cells and Tregs.

Taken together, a new mouse model could be established in this project that mirrors the condition of allergic patients under AIT better than traditional murine models of AIT. In this novel mouse model (and in the traditional AIT-model), the combination of IL-4 receptor inhibition with allergen-specific immunotherapy did not immediately improve the clinical outcome. This could be either caused by the strong impact of AIT alone that makes further significant changes very difficult to observe, or by the choice of

the mouse model in which tolerance induction might be induced by other mechanisms than in human patients. Of course, it is also possible that the chosen approach simply does not lead to an additional therapeutic benefit. Further experiments in other mouse models are necessary to finally clarify the remaining questions. Independent from the effect of IL-4 Mutein, the detailed analysis of AIT during allergen exposure gave interesting new insights into AIT induced immunological changes. The most prominent newly discovered effect of AIT alone was its suppressive effect on ST2-expression of T helper cells. The importance of ST2-downregulation for an improved tolerance needs to be elucidated.

Additionally, it is free of doubt that the observed IL-4 Mutein-related changes on T-helper cell level should also be further investigated: Firstly, the inhibitory effect of IL-4 Mutein treatment during AIT on the recently identified sub population of $GATA3^+$ $FoxP3^+$ T cells is of great interest, as these cells, which are also named "ex-Tregs", have been shown to be strong TH2 cytokine producers. Secondly, more research needs to be done on the impact of IL-4 Mutein on $FoxP3^+$ $ST2^+$ cells that were strongly decreased by AIT in presence of IL-4 Mutein. These cells have also been linked to TH2 cytokine production. Both populations seem to play a decisive role in the balance of Tregs and TH2 cells. This makes them especially exciting new therapeutic targets. Furthermore, the observed unexpected effect of IL-4 Mutein treatment on $Ror\gamma t^+$ $CD4^+$ T cells should be further clarified.

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