Investigation of Immune Responses in Different Mouse Models of Allergic Asthma

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by

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Declaration

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Frank Kirstein

February 2008

In loving memory of my mother Gisela Kirstein

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List of Abbreviations and Symbols

AAM	alternatively activated macrophage
Ab	antibody
Ag	antigen
AHR	airway hyperesponsiveness
Alum	aluminium hydroxide
AMCase	acidic mammalian chitinase
Ani s 1	*Anisakis* allergen 1
AP	alkaline phosphatase
APC	antigen presenting cell
APES	3-aminopropyltriethoxysilane
Arg-1	arginase-1
ASMC	airway smooth muscle cell
BAL	bronchoalveolar lavage
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAM	classically activated macrophage
CD	Cluster of Differentiation
CO_2	carbon dioxide
Cre	Cre recombinase
DAB	diaminobenzidine
DC	dendritic cell
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced Chemiluminescence
ECP	eosinophil cationic protein
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
FACS	fluorescence-activated cell sorting
FcεRI	high affinity IgE receptor
FcγrII	IgG receptor II
FcγRIII	IgG receptor III
FCS	Foetal Calf Serum

Fel d 1	cat (feline) allergen 1
FDA	Foods and Drugs Administration
Fig.	Figure
FITC	Fluorescein isothiocyanate
Fizz1	found in inflammatory zone 1
g	gram(s)
HRP	horseradish peroxidase
H&E	haemotoxylin and eosin
H_2O	water
H_2SO_4	sulfuric acid
ICS	inhaled corticodteroids
Ig	immunoglobulin
IFN	interferon
IL	interleukin
IL-13Rα1	interleukin 13 receptor alpha 1
IL-13Rα2	interleukin 13 receptor alpha 2
IL-4Rα	interleukin-4 receptor alpha
IMDM	Iscove's Modified Dulbecco"s Medium
i.n.	intranasal
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IRS	insulin receptor substrate
IVC	individually ventilated cages
kDa	kilo Dalton
ko	knockout
L	litre
lox	loxP recognition site
LPS	lipopolysaccharide
LysM	Lysozyme M
L_1	first-stage larva
L_2	second-stage larva
L_3	third-stage larva
L_4	fourth stage larva
M	molar
MBP	major basic protein
MCh	methacholine

MHC	major histocompatibility complex
min	minutes
mg	milligram
mg/ml	milligrams per millilitre
ml	millilitre
mM	millimolar
MMCP-1	mouse mast cell protease-1
mMGL	mouse macrophage galactose-type-C-type lectin
mol	moles
n	number
ND	not determined
ng	nanogram
ng/ml	nanograms per millilitre
NK	natural killer
nm	nanometre
NO	nitric oxide
iNOS	inducible nitric oxide synthase
OCT	oxacalcitriol
OD	optical density
OVA	ovalbumin
P	probability
PAMP	pathogen-associated molecular pattern
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Penh	enhanced pause
PPARγ	peroxisome proliferator-activated receptors
PRR	pattern-recognition receptor
RAST	radioallergen sorbent test
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
RNA	ribonucleic acid
rcf	relative centrifugal force
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMC-MHC	smooth muscle cell-myosin heavy chain

SNP	single nucleotide polymorphism
SPF	specific pathogen free
spp	species (plural)
SPT	skin prick test
STAT	signal transducer and activator of transcription
TARC	thymus- and activation-regulated chemokine
TCR	T cell receptor
TGF-β	transforming growth factor beta
Th	T helper cell
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	toll-like receptor
TMB	3.3', 5.5'-tetramethylbenzidine
Treg	T regulatory cell
UCT	University of Cape Town
U.K.	United Kingdom
U.S.A.	United States of America
VEGF	vascular endothelial growth factor
WAO	World Allergy Organisation
WBT	whole body plethysmography
WHO	World Health Organization
WT	wildtype
x	times
α	alpha
β	beta
γ	gamma
γc	common gamma chain
μg	microgram
μg/ml	micrograms per millilitre
μl	microlitre
μm	mirometer
^{0}C	degrees celsius
%	percent
<	less than
-/-	knockout

Abstract

Allergies are a common chronic disease and considerably decrease the quality of life for affected individuals. Understanding the immune responses during allergic diseases is essential for both diagnosis and the development of effective therapies. The route of sensitisation to allergens is one factor that influences the immune response and the outcome of allergic diseases and both human and animal studies have highlighted IL-4Rα as an important component in the induction of allergy. The aim of this study was to investigate the contributions of the route of sensitisation to allergens with focus on the significance of cell specific expression of IL-4Rα in the onset of allergy.

The route of sensitization to *Anisakis pegreffii* influences the outcome of experimental allergic asthma

Worldwide, increasing numbers of allergies to the fish parasite *Anisakis pegreffii* are reported. *Anisakis* can cause allergies after accidental infection of humans and in the occupational environment. Currently it is not clear if different exposure routes to *Anisakis* affect the development of allergic asthma and if they have an influence on the immune response. To address these questions, the present study investigated immune responses and disease development after *Anisakis* live infection and after nasal sensitisation in a mouse model of allergic airway disease. We showed that the route of sensitisation influences the outcome of *Anisakis pegreffii* induced allergic asthma and demonstrated important contributions of IL-4Rα to the underlying immune response.

Alternatively activated macrophages are not necessary for the development of experimental allergic lung inflammation

Development of alternatively activated macrophages (AAM) is induced by signals of IL-4Rα. Alternatively activated macrophages (AAM) are a feature of allergic asthma in clinical and experimental investigations but their role in the development of allergy is not defined. To address this, a model of acute allergic

asthma was used to compare mice deficient in AAM ($LysM^{cre}$IL-$4R\alpha^{-/lox}$ mice) with control mice. We found that the presence of AAM at early stages of allergic airway inflammation these cells was not required for the onset of the disease.

Smooth muscle IL-4Rα is not required for experimental allergic asthma

In vitro studies have suggested that IL-4Rα signalling on airway smooth muscle cells (ASMC) is critical for airway inflammation and airway hyperresponsiveness. Using mice deficient for IL-4Rα in ASMC, the *in vivo* effects of impaired IL-4Rα signalling in ASMC on the outcome of asthmatic disease were investigated. The impairment of IL-4Rα on SMC had no effect on major aetiological markers of allergic asthma. These findings suggest that IL-4Rα responsiveness in airway SMC during the acute phase of allergic asthma is not critical for the outcome of the disease.

Conclusions

The present study showed the importance of the route of sensitisation and IL-4Rα in the development of allergy to *Anisakis pegreffii*. The use of *in vivo* models of experimental allergic asthma revealed that the route of sensitisation can influence the underlying immune response of the disease. Furthermore by using mice with cell specific deficiencies in IL-4Rα it was demonstrated that expression of this receptor on smooth muscle cells and macrophages is not essential for the development of acute experimental allergic airway disease, as it has been previously suggested.

Chapter 1

General Introduction

1 General Introduction

1.1 Allergy-General Overview

1.1.1 Prevalence of allergies

Allergy represents one of the most prevalent chronic diseases worldwide with affected individuals having considerably reduced quality of life. The number of reported allergies has increased worldwide in the last decades, and it is estimated that 20% of the world's population is affected by various forms of allergic diseases, though the prevalence varies considerably between different countries.[1] The reason for these regional differences is not understood likely to be affected by an individual's life style and genetic predisposition.

A worldwide study in 1998 on the prevalence of allergic symptoms in children showed a variation from 1.4 to 39.7% between different study centres.[2] A follow- up study reported a rise in prevalence in most places within 7 years.[3] In South Africa, allergic prevalence is currently estimated 15-20% in children and was increasing over the last years[3]. As allergic diseases are predominantly reported in developed countries they might be connected with a "westernized" way of living.[4] In rural communities allergic diseases appear to be less common, while urban environments appear to increases the risk of developing allergies.[5, 6] In twenty European countries, large variations in allergy prevalence have been shown between study centres and it remains difficult to explain these observations.[2, 3]

Figure 1. World map showing direction of change in prevalence of asthma symptoms for children from 13–14 years. Prevalence in South Africa increased over the last years. Each symbol represents a study centre. Blue triangle=prevalence reduced. Green square=little change. Red triangle=prevalence increased. From Asher, 2006.[3]

There is a strong hereditary component in the disposition to allergic diseases. Children with allergic parents or a family history of allergies have an increased risk of developing allergies. The role of genetic variances in allergic disease has been recognised for some time and polymorphisms in over 70 candidate genes have been discovered, many of them involved in immune responses.[7] Whereas the link between allergies and genetic factors may contribute to regional differences in the prevalence of allergies, it does not explain its increasing trends in many parts of the world. Genetic susceptibility also seems to be influenced by environmental factors such as exposure to pollutants or allergens which have different effects on different genotypes.[8]

An explanation for the influence of environmental factors is the hygiene hypothesis, which was first described in 1989.[9] This hypothesis is based on epidemiological studies which show that exposure to infections in early childhood reduce the risk of developing allergies.[10] Immunological mechanisms to explain this hypothesis conclusively are still missing.[11, 12] It has been suggested that early allergen exposure in the presence of bacterial

infections or microbial components induces the development of a protective Th1 type immune response to the allergen. This prevents a Th2 type and therefore allergic immune response.[11] Another mechanism might be the development of suppressive immunoregulatory responses to chronic or repeated infections.[13] Practical implications from the hygiene hypothesis for the prevention and treatment of allergies are limited and an association between vaccinations or antibiotic usage, as sometimes suggested in the media, has not been shown.[14, 15] Some authors also questioned the validity of the epidemiological studies.[16, 17] However, the hygiene hypothesis can be useful as a guideline for future epidemiological or immunological studies into causes and prevalence of allergies.[18]

1.1.2 Definition of Allergy

Allergy is an immunological disorder where the balance of the defence mechanisms between protection and overreaction is disturbed. The term allergy was introduced in 1905 by von Pirquet to describe a situation where an external agent causes some form of changed or altered response in the recipient which could be either immunological protection or hypersensitivity.[19] Over time this definition has changed and today allergy is only used in the latter meaning.[20] The definitions used here are recommended by the World Allergy Organisation (WAO) and the World Health Organisation (WHO) with the intention of harmonising the nomenclature of allergic disorders.[21, 22]

Hypersensitivity reactions are reproducible symptoms in response to substances at doses tolerated by normal individuals. The term allergy is restricted to hypersensitivity reactions mediated by allergen specific immunological mechanisms.[22] The immune response in allergies is either antibody or cell mediated. In the majority of cases, allergen specific IgE antibodies induce the allergic response. Non-IgE mediated allergic reactions can be triggered by IgG antibodies as in anaphylaxis, or by allergen specific lymphocytes, as in contact urticaria or non-atopic asthma.[22-24] The tendency to become sensitised to common allergens by producing high levels of

specific IgE antibodies is called atopy. Atopic individuals are therefore predisposed for the development of allergic symptoms and the diagnosis of atopy requires proof of specific IgE antibodies in the blood serum.

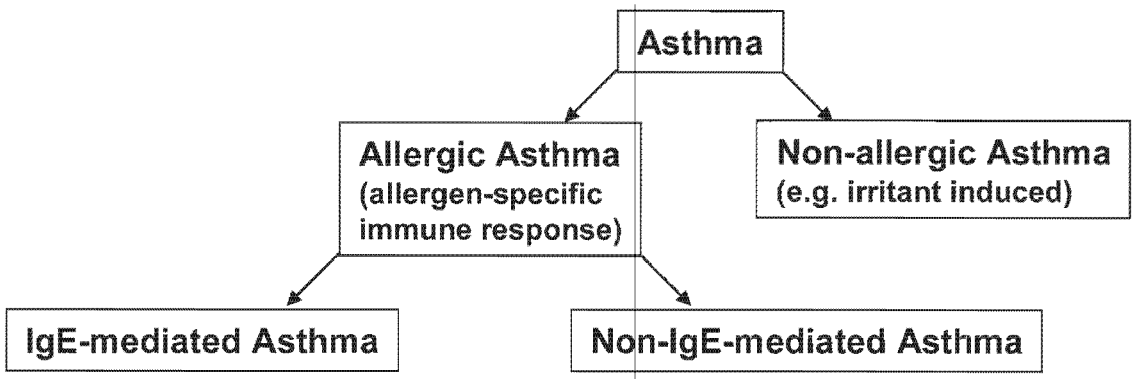

Figure 2. Allergic and non-allergic asthma. Asthma resulting from immunological reactions should be called allergic asthma. Most cases are initiated by IgE antibodies, but non-IgE mediate asthma has been reported. Adapted from Johansson, 2004.[22]

Hypersensitivity reactions have been classified by Coombs and Gell into type I-IV reactions [25] and this classification is still widely used. Typical IgE mediated allergies are immediate type I reactions under this classification, characterised by the release of proallergic mediators such as histamine, leukotrienes and prostaglandins from mast cells and basophils. However, besides IgE antibodies, chronic effects and other components of the immune system, such as T cells, are involved in the pathogenesis of allergies and the usefulness of this classification for describing allergic reactions has been questioned.[26]

This review will focus on IgE-mediated allergies with special emphasis on allergic asthma as one of the most prevalent and severe disease manifestations.

1.1.3 Symptoms of allergic diseases

Allergic diseases affect individuals in different ways and symptoms vary considerably depending on the target organ. Manifestations of allergies include conjunctivitis, rhinitis, asthma, dermatitis, urticaria and anaphylaxis and often occur in different combinations. The reason why patients develop only certain signs of allergy is not understood and manifestations may change with age or remit completely.

Skin manifestations of allergic diseases are dermatitis and urticaria. Both are accompanied by pruritus and a typical sign of dermatitis is eczema, with hives and wheals being a sign of urticaria.

Symptoms of allergic rhinitis are sneezing, itching, increased nasal discharge and often itching of the palate and inner ear. Conjunctivitis with running and itching eyes is found together with rhinitis in many cases. Both manifestations are often seasonal, e.g. in pollen allergies but can also be perennial, especially if indoor allergens are involved.[27]

A more severe manifestation of allergy of the respiratory tract is allergic asthma, which often occurs together with allergic rhinitis.[28] Allergic asthma is responsible for 80% of childhood and over 50% of adult asthma.[1] Shortness of breath, wheezing and cough in consequence of bronchoconstriction and excessive mucus production are normally transient, but patients can develop chronic inflammation of the airways after long term allergen exposure.

Anaphylaxis is a severe systemic response occurring usually within minutes after exposure to allergens and is fatal in approximately 1% of cases.[29] Skin, cardiovascular, respiratory and gastrointestinal systems can be affected and patients may go into anaphylactic shock due to a sudden collapse of the respiratory and/or circulatory system.[27]

1.1.4 Allergens

The general definition of an allergen is an antigen that causes allergic diseases,[22] normally an IgE-mediated allergic disease. According to a standardised nomenclature that has been proposed by the WHO, allergens are designated according to the species name of their source as follows: first three letters of the genus name, first letter of the species and a number (e.g. Fel d1 for house cat *Felis domesticus*).[30]

Allergens are in most cases proteins that are able to induce an IgE memory response, but specific features that distinguishes them from non-allergenic proteins are not defined.[31] The particle size may be important for the kind of allergic response that is induced by a specific allergen. Large particles, such as grass pollen, may not be able to enter the lungs and cause allergic asthma, but are rather deposited in the nose or throat where they may induce allergic rhinitis.[32]

No structural characteristics of allergens are defined and every protein that is able to reach and stimulate immune cells may be able to induce an allergic response.[33] Protease activity has been described for some allergens, such as the house dust mite allergen Der p1.[34] Enzymatic activity was associated with increased inflammatory responses,[35-37] but enzymatic activity is not a common feature of allergens, and enzymes in general are not more allergenic.[32] Characteristics of some food allergens are their resistance to enzymatic digestion and processing, high abundance in the food, and several linear IgE binding Epitopes.[38]

The observation that allergens show cross-reactivity with other allergens, sometimes from very different sources, has initiated bioinformatical approaches to identify common structural features of IgE binding of allergens.[39] More information about three-dimensional structures [40] and binding Epitopes [41-43] of allergens is becoming available and may enable better prediction of allergenicity and crossreactivity of proteins in the future.[44]

1.1.5 Routes of exposure to allergens

Most allergens in IgE mediated diseases enter the body by inhalation or ingestion, but some may also be injected, such as insect venoms or drugs. Aeroallergens can occur seasonally, such as grass and tree pollen, or perennially, such as dust mite, moulds or domestic animal allergens. The appearance of allergic symptoms at certain times of the year is often the first indication of the nature of the allergen. Allergic rhinitis and asthma, but also atopic eczema, are the most common symptoms in response to aeroallergens.[27]

Development of allergic reactions is also observed after exposure to aerosolised allergens in the work place. Symptoms of IgE-mediated occupational allergies are allergic rhinitis, asthma or urticaria, whereas contact dermatitis is mostly non-IgE, but T-cell mediated.[22,45,46,47] In the occupational environment it is important to distinguish between immunological mediated reactions and reactions that are irritant-induced, as this has implications on the treatment and prevention strategies. To distinguish between immunological occupational asthma and irritant-induced occupational asthma, several criteria have to be considered. Airway hyperresponsiveness, onset of asthma after workplace exposure, association of asthmatic symptoms with the work exposure and induction of symptoms by the suspected allergen at the workplace are required for the diagnosis of occupational allergic asthma.[48]

Food allergies occur after ingestion of allergens and can be distinguished from food intolerance by their immunological mechanism, which mostly involves IgE mediated responses. Symptoms of food allergy can involve the skin, the respiratory tract and the gastrointestinal tract. Skin manifestations, such as eczema and urticaria, often include the lips mouth and throat. Nausea, vomiting and diarrhoea are common in food allergies, whereas rhinitis and asthma are less frequently seen. Anaphylaxis is the most serious complication

in food allergies and the allergen sources in fatal cases were mostly peanuts and tree nuts, but also milk, egg and seafood.[49]

Small amounts of food allergens can be sufficient to induce a severe allergic reaction and patients might not be aware of the food containing allergens. Hidden food allergens are often unintentionally introduced during processing and preparation of food, and their content may be below the legal requirement for labelling. Milk, eggs, fish, crustaceans, shellfish, peanuts, tree nuts, wheat, and soy are common hidden food allergens and legislation requires declaration in Europe and North America, even if only small amounts are found in the food.[50]

Food allergens may also become aerosolised during processing and cooking, and can induce allergic reactions after inhalation. This is of particular importance in the occupational environment where 10% of asthma is induced by aerosolised food.[51] In seafood processing industries, workers are exposed to allergens during cleaning, cooking or fish meal production and the prevalence of allergic symptoms ranges from 3-36%.[48]

Parasites hidden in food are another source of allergens that has long been underestimated. *Anisakis spp.* is a group of nematode parasites frequently found in fish, molluscs and shellfish that are designated for human consumption.[52] The species most commonly found in seafood, *A. simplex* and *A. pegreffii*, have a worldwide distribution and are closely related to the human parasite *Ascaris lumbricoides*.[52, 53] After accidental infection with the live L3 larvae, humans develop a gastrointestinal zoonosis named anisakiasis, characterised by abdominal pain, nausea or vomiting, that is often misdiagnosed as gastric ulcer.[54] During *Anisakis* infection, IgE mediated hypersensitivity reactions may occur [55, 56] and *Anisakis* related allergies have also been reported after consumption of fish contaminated with dead parasites.[57] In regions with high fish consumption, *Anisakis* is an important inducer of allergic reactions and was the most frequent cause of food-related anaphylactic reactions in a recent study in Spain.[58] Hidden *Anisakis* allergens are also an important factor in occupational environments. A recent study in South African on fish processing workers revealed a high prevalence of

sensitisation to *Anisakis* allergens, that was associated with allergic symptoms [59] and several cases of occupational *Anisakis* allergies have been reported from Spain.[60, 61]

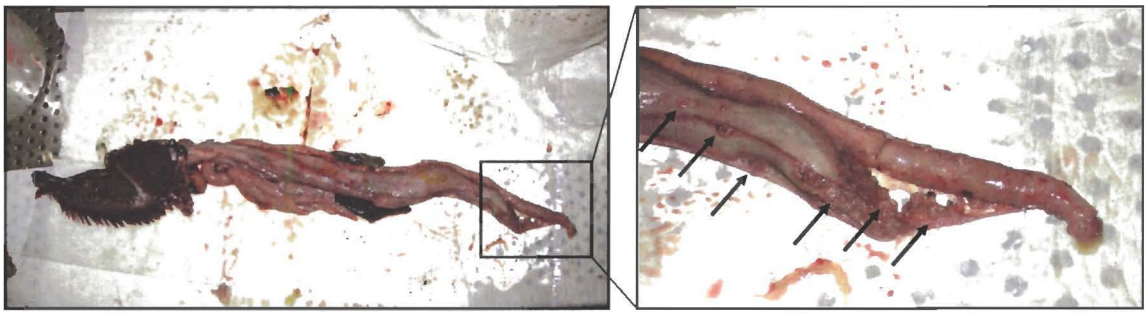

Figure 3: Visceral organs of *Thyrsites atun* (snoek) infected with *Anisakis pegreffii* L3 larvae. Most parasites live in the visceral cavity (arrows) but few individuals may migrate into the muscle of the host. Humans might get exposed to Anisakis allergens after consumption or processing of contaminated fish.

1.1.6 Diagnosis of allergy

For the diagnosis of an allergic disease it is essential to prove an immunological mechanism behind the symptoms and to identify the allergen to which the patient is reacting.[22] For the medical history of an allergic disorder, it is important to find out about the symptoms and the situation when the symptoms occur, in order to identify the allergens to be considered for more specific tests.

Most allergies are mediated by allergen-specific IgE, and their detection is important for diagnosis. Usually, the first recommended test is a skin prick test (SPT) where a small amount of allergen is applied onto the skin. This induces the release of inflammatory mediators by mast cells and provokes a skin reaction if the patient is sensitised to the allergen. Allergens can also be applied by subcutaneous injection or scratch test.[27] Diagnosis of food allergies is complicated by the fact that SPT results are often unreliable and may give false positive results if the SPT reaction is weak. Here a double-blind, placebo

controlled food challenge (DBPBFC) under close supervision is recommended.[62, 63]

In vivo diagnostic tests are fast, cheap and mostly reliable tests, but have some disadvantages that need to be considered. These methods bring the risk of anaphylactic reactions after allergen application onto the skin and are not reliable if the patient is having anti-allergic medication. Standardised allergen preparations are often not available for new or uncommon allergens, or it might be difficult for the patient to report to a specialised surgery.

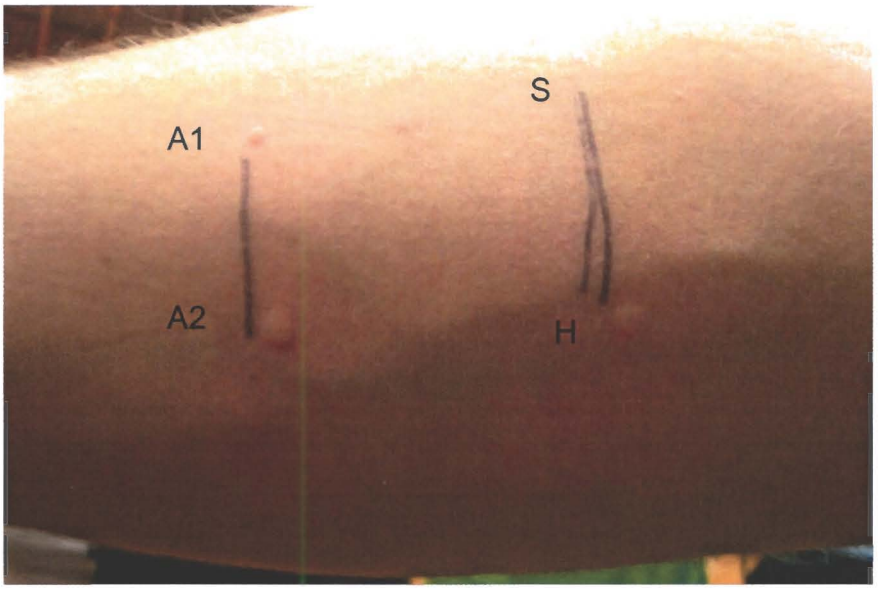

Figure 4. Positive skin-prick test. A1/2: allergen preparations;
S: saline (negative control; H: histamine (positive control)

Several *in vitro* methods are available for allergy diagnostic in blood samples. Commercially available systems allow measurement of allergen specific IgE concentrations in small blood samples by Radioallergosorbent test (RAST) or Enzyme linked immunosorbent assay (ELISA). Basophil activation assays measure mediator release such as histamine or leukotrienes after *in vitro* stimulation with allergens.[27] These methods are particularly useful for testing self-made allergen preparations or non-purified samples containing allergens from workplaces [64] as they bring no risk of adverse reactions for the patient.

Diagnosis of allergic asthma requires the proof of airway hyperresponsiveness (AHR) in addition to the general allergy tests. Changes in airway resistance in response to unspecific bronchodilators such as methacholine are indicators of AHR and can be measured by spirometry or whole body plethysmography (WBP).[27]

Diagnosis of *Anisakis* allergies mostly relies on specific IgE determination by ELISA or Western Blot. Hypersensitivity reactions due to live infection with *Anisakis* larvae are a combination of parasite infection with allergic reaction, and diagnosis in this special case can be performed by endoscopic examination and identification of the worm.

1.1.7 Therapy of allergic diseases

The first step in developing allergic diseases requires the sensitisation to an allergen, usually characterised by establishment of an IgE memory response. As discussed earlier, the reason why certain people get allergies is poorly understood and seems to be dependent on genetic and environmental conditions as well as the personal history of the patient. Therefore no strategies are known so far that can prevent allergic sensitisation but minimising risks is recommended by avoiding exposure to potential allergens at home or at the workplace, especially when a family history of allergy exists. When sensitisation to an allergen has been diagnosed, avoiding exposure is the first step preventing establishment of allergic symptoms.

Allergen immunotherapy is an effective treatment for IgE-mediated allergic rhinitis and asthma [65] and is the only currently available strategy for curing allergic diseases rather than treating symptoms. This is achieved by repeated administration of allergen preparations over a long period, often several years. Subcutaneous injection has been the preferred way of administration but sublingual protocols have been developed to reduce the risk of severe side-effects and to improve efficiency.[65] The principle of immunotherapy relies on restoring a normal immune response in the patient that tolerates allergens.

After immunotherapy, patients have increased levels of allergen-specific IgG antibodies that may prevent binding of IgE to allergens. Numbers of effector cells such as mast cells and basophiles were reduced and an increase in allergen specific regulatory T cells as well as a switch from a Th2 type to a Th1 type immune response was observed.[66] Immunotherapy is not efficient in all patients, especially when they have developed allergies against multiple allergens or show perennial symptoms.[67]

Several pharmacological strategies exist for allergy treatment and its efficiency is often dependent on the type of symptoms and personal response to the drugs.

Antihistamines are the first medication of choice because of their efficacy, rapid onset, long duration of activity and low side effects. They are selective antagonists of the H1 histamine receptor thereby inhibiting the inflammatory effects of histamines, an important mediator in allergic diseases. Second generation antihistamines, which lack the sedative effects of first generation drugs, are an effective treatment, especially for allergic rhinitis [68] and urticaria. Antihistamines reduce asthma symptoms [69, 70] but are not considered an effective asthma treatment.[71]

Leukotriene inhibitors are a relatively new class of allergy drugs and are leukotriene receptor antagonists. They have been shown to be efficient in asthma and allergic rhinitis therapy with low risk side effects.[71-73]

Humanised anti-IgE monoclonal antibody was effective especially in patients with severe, poorly controlled asthma, and might be a new treatment option for this patient group.[74] Treatment with anti-IgE antibody reduced inflammatory cell numbers in the lung and nose and circulating IgE in the blood of patients with allergic rhinitis and asthma, which correlated with an improvement of symptoms.[75]

Treatment of allergic asthma requires special medication targeting the airway symptoms. β2 adrenergic receptor agonists are widely used for the immediate treatment of acute asthmatic symptoms, and their activity as bronchodilators relies on induction of smooth muscle cell relaxation. Progress has been made

in reducing side effects by improving specificity for the β2 receptor and increasing the duration of activity.[71]

For the long term control of asthma, inhaled corticosteroids (ICS) are the most effective treatment currently available. Prolonged treatment on a scheduled basis leads to sustainable improvement of lung function and asthmatic symptoms [76] by controlling the underlying inflammation in the airways.[77, 78] The introduction of ICS has significantly reduced the side effects of systemic corticoid treatment which has limited their usefulness in allergy treatment for a long time.[71]

Currently available treatments are effective in controlling a wide range of allergic symptoms but they are not able to cure allergic diseases, and symptoms recur when intake is stopped. Therefore a better understanding of the mechanisms behind allergies is essential for the development of effective treatments. The development of new technologies for fast and cheap detection of single nucleotide polymorphisms (SNP) in individual genomes will make the detection of disease associated genes in individual patients possible [79] and allow the development of personalised therapeutic strategies for allergies and asthma.[80]

1.1.8 Immunomodulatory strategies in allergy therapy

A better understanding of immune responses in the development of allergies has led to several immunomodulatory approaches for new therapeutic strategies.[81] In most cases, antagonists for cytokines which were believed to play important role in allergy development were tested in clinical trials.

Interleukin (IL-)-5 is an important effector molecule for the differentiation and recruitment of eosinophils.[82] As eosinophils are associated with allergic diseases it was believed that these cells are important in disease development. Administration of monoclonal anti-IL-5 antibodies reduced numbers of eosinophils, but this had only little effect on symptoms of allergic

asthma and dermatitis. However, the efficiency of this antibody is still being investigated in long term studies. Strategies that targeted IL-4 also did not bring considerable improvement in allergic symptoms and this approach is no longer followed. Results of anti-IL-4 and anti-IL-5 trials also led to the conclusion that these molecules as well as eosinophils might not be crucial in allergy development.[83]

There is evidence from animal models [84-86] and human studies [87] that IL-13 has an important role in the pathogenesis of allergies and asthma. AHR, mucus production and eosinophilic inflammation are dependent on IL-13 or IL-4Rα, the common part of IL-4 and IL-13 receptors.[88] Several monoclonal anti-IL-13 antibodies and IL-4Rα antagonists are currently being tested in clinical trials.[89]

Blocking of TNF-α, which is also associated with asthmatic diseases, has resulted in considerable improvement of symptoms in clinical trials and might be a promising target for at least a subset of patients with severe asthmatic diseases.[90]

Other immunomodulatory strategies include administration of toll like receptor (TLR) agonists like LPS or CpG-DNA in order to shift the cytokine response from an allergy associated Th2 response to a protective Th1 response.[89]

Most of the immunomodulatory therapy strategies are still in trial phases and their efficiency and safety for the treatment of allergic diseases remains to be proven. As allergies are complex diseases with a large variety of manifestations, many therapies may only be efficient in a subset of patients. The immune system also shows a degree of redundancy, and targeting a specific molecule might not help, due to compensation by other parts of the immune response. Therefore, further research on the immune responses underlying allergic diseases is important for the development of efficient and safe therapies.

1.2 Allergy-Immunology of allergic responses

1.2.1 Route of sensitisation influences the immune response in allergy

In allergy, the immune system generates a response to harmless, non-infectious antigens that are tolerated by healthy individuals. It is important to understand the immunological mechanisms that are responsible for this disregulated immune response in order to provide effective therapies and diagnostic tools for allergic patients.

The route of sensitisation to allergens is one factor that influences the immune response and the outcome of allergic diseases. The magnitude of IgE responses to allergens has been shown to be dependent on differences in the route of sensitisation.[91-93] In one study, aerosol exposure and dermal sensitisation induced a stronger IgE response than intraperitoneal (i.p.) sensitisation and severity of disease symptoms correlated with antibody titres.[94] Development of AHR in the absence of IgE was found only after i.p. allergen sensitisation, but not after nasal sensitisation in animal models of allergic airway disease.[95, 96] The route of sensitisation to birch pollen influenced antibody and cytokine responses in a mouse model, with production of the cytokines IL-5 and IL-13, but not IL-4, dependent on the route of sensitisation to the allergen.[92]

In *Anisakis* allergy, the influence of the route of sensitisation is of particular interest as sensitisation can occur after infection with the live parasite. *Anisakis* is an unusual case where a parasitic infection may induce allergic sensitisation, whereas other nematode infections have been associated with protection from allergic reactions.[13, 97] It is still controversial whether contact with *Anisakis* proteins is sufficient for allergic sensitisation, or if live infection is required.[56, 98] Clinical studies with *Anisakis* allergic patients gave conflicting results.[99, 100] A recent study in our laboratory demonstrated an increased risk of sensitisation to *Anisakis* that correlated with allergic airway symptoms after

exposure to aerosolised proteins in the occupational environment. This study also demonstrated that mice show allergic airway symptoms after live infection and oral protein challenge.[59] In the animal study, the immune response and severity of allergic symptoms was dependent on the route of sensitisation, with more severe symptoms after live infection.

It is apparent from these studies, that the route of sensitisation has an important influence on allergen specific antibody and cytokine responses and on the outcome of allergic diseases. The immunological mechanisms behind these observations are not entirely clear and further investigations of the immune responses after different means of sensitisation to *Anisakis*, and of the general immune mechanisms in allergy and asthma are required.

1.2.2 Innate and adaptive Immunity

Immune responses are mediated by leukocytes that provide innate and adaptive mechanisms of protection. The innate immune response is the first line of defence against invading pathogens and has cellular and humoral components. The latter consist of plasma proteins of the complement system that can bind to invading microorganisms. Complement proteins can kill pathogens directly by cell lysis or they mark them for phagocytosis by immune cells, a process called opsonisation.[101]

Phagocytotic cells, such as macrophages and neutrophils, recognise opsonised particles by complement receptors, but also by pattern recognition receptors (PRR), that are specific for characteristic pathogen-associated molecular patterns (PAMPs). Several PRR are expressed by immune cells, e.g. toll-like receptors, mannose receptor or C-type lectins, and allow a specific recognition of invading pathogens.[101] In contrast to the receptors of the adaptive immune system, they do not change their antigen specificity. Natural killer (NK) cells are specialised cells of the innate immune system for identification and killing of virus infected cells or tumour cells. Pathogens that are too large for phagocytosis, e.g. nematodes, can by attacked by

eosinophils by their release of cytotoxic proteins, such as eosinophil peroxidase (EPO), major basic protein (MBP) or eosinophil cationic protein (ECP).[102] Mast cells and basophils, upon activation, release inflammatory mediators such as histamine, prostaglandins or leukotrienes and are directing other leukocytes to the site of inflammation by cytokine release.[103] Mast cells are long living cells that can be found in all tissues whereas basophils are circulating blood leukocytes.[103] The innate immune system provides several effective mechanisms for controlling invading pathogens. In case the innate immune mechanisms are not sufficient for clearing the infection, the adaptive immune defence gets activated.

The adaptive immune response provides an effective and specific protection against invading pathogens and creates and immunological memory, that can get activated in short time after reinfection. Characteristic for adaptive immunity is the generation of antigen receptors in a random process in T and B lymphocytes, which are virtually able to recognise all invading pathogens. Antigen presenting cells (APC), including dendritic cells and macrophages, are essential in this process. They present antigen-peptides on MHC-II molecules on their surface to $CD4^+$ T-helper (Th) cells. Recognition of the peptide-MHC-II complex by the T cell receptor induces clonal expansion and activation of the Th cell. APC provide further signals to Th cells by the release of cytokines and expression of co-stimulatory molecules on their surface, and influence the development of Th1 or Th2 type immune responses. These cytokine signals are dependent on preceding innate responses and APC are an important link between innate and adaptive responses. After activation, Th cells start cytokine expression and induce the generation of a specific antibody response by activating B cells that recognise the same antigen. Depending on co-stimulatory cytokine signals, B cells produce several different types of antibodies, i.e. IgM, IgG, IgE, IgA or IgD. After activation by T cells, B cells can differentiate into antibody producing plasma cells or memory B cells. Together with memory T cells, they provide an immunological memory that is able to launch a rapid response after at the next encounter with the antigen.[101]

Adaptive immune responses need to be tightly controlled in order to prevent development of immune responses against non-pathogenic antigens. The random process of antigen receptor generation may produce receptors against self-antigens that need to be eliminated in order to prevent autoimmune diseases. Furthermore, control mechanisms are necessary to prevent immune responses against foreign, but harmless antigens, such as allergens.

1.2.3 Cytokines

Adaptive and innate immune responses require interactions of different types of immune cells as well and resident cells. Essential in the regulation of immune responses are cytokines, small messenger proteins that are produced by various cells in response to a number of stimuli and can be recognised by any cell expressing the appropriate cytokine receptor. Cytokine production is transient and they regulate differentiation, proliferation, activation or recruiting of cells. Their mode of action can be autocrine, by binding on receptors on the same cell that secreted it, or paracrine, by binding on other cells in close proximity.[88] Cytokines include several groups of signalling molecules, including interleukins (IL), chemokines and interferons (IFN). Theses names, given at the time of their discovery were used to describe their functions or target cells (e.g. interleukin: cytokine acting between two leukocytes) but increasing knowledge about their functions revealed a more complicated situation and one cytokine may be produced in and recognised by a variety of different cell types where they induce different reactions.[88]

1.2.4 Differentiation of T-helper (Th) subsets

Allergic responses are associated with Th2 type immune responses which are also induced by infections with parasitic helminths. The term originated from different types of $CD4^+$ T-helper (Th) cells that were first described in mice [104]

as Th1 and Th2 cells according to differences in the cytokine profile expressed after stimulation. Th1 and Th2 polarised cells have later been identified in humans and seem to have similar properties and implications on the immune response [105]. Since the present study investigated mouse models of allergic airway responses, this review is focused on the murine immune response.

The cytokines produced by the different T helper cells are, among others, IFN-γ for Th1 cells and IL-4, IL-5 and IL-13 for Th2 cells.[106] These cytokines were subsequently named as Th1 and Th2 cytokines respectively. A third T-helper subset are Th17 cells, secreting IL-17A and IL-17F.[107]

Th1 type immune responses normally develop after microbial infections and intracellular pathogens whereas Th2 type responses are induced by extracellular parasites, such as nematodes or trematodes, but also in allergic reactions.[88] Th1 and Th2 type responses can be distinguished by their antibody profiles. Th1 cells, by secretion of IFN-γ, induce production of IgG2a and IgG2b antibodies in B cells, whereas Th2 cells induce the production of IgE and IgG1.[108] IL-13 signals are important for the class switch to IgE, as IL-13 receptor deficient mice show an impaired IgE response, whereas IL-4 may be responsible for the production of IgG1.[109] Th17 functions are not as well defined, but are essential for clearance of several microbial infections,[110-112] and are also associated with asthmatic symptoms [113-116] and autoimmune diseases.[117-119]

Differentiation of naïve T cells into distinct T-helper cells subset is dependent on the signal strength, nature of the antigen presented by APC[120,121] and the cytokine environment. For development into Th1 cells, IL-12 and subsequent IFN-γ signalling is essential, and IL-18 provides further signals for terminal differentiation.[122] IL-4 is crucial for Th2 development and induction of a Th2 type immune response.[123, 124] Differentiation of naïve T cells into Th17 cells requires IL-6 together with TGF-β, and IL-23 promotes Th17 effector functions.[125, 126] There are reciprocal regulations between the three different T

helper cell types. In the presence of IL-4 and IFN-γ the development of Th17 cells is inhibited, whereas IL-4 alone inhibits Th1 differentiation and IFN-γ alone inhibits Th2 differentiation.[127-130]

T regulatory (T_{reg}) cells are a fourth subset of $CD4^+$ T cells, which are characterised by expression of CD4, CD25 and the transcription factor Foxp3.[131] These naturally occurring $CD4^+CD25^+Foxp3^+$ T_{reg} cells are essential for controlling self tolerance and inflammatory immune responses and are a distinct T cell lineage.[132] The mechanisms of immunosuppression by T_{reg} cells are not completely understood, but direct cell contact, IL-10 and TGF-β production seem to be involved.[133]

1.2.5 Immune response in allergy and asthma

Allergy is an inflammatory disease caused by a Th2 type immune response. As discussed earlier, the reasons for the induction of this response to otherwise harmless allergens in susceptible individuals are poorly understood and genetic factors seem to play an important role. This is supported by the observation that different inbred laboratory mouse strains also show the tendency of developing either a Th1 response (e.g.C57/Bl6 mice) or a Th2 response (e.g. Balb/c mice).

During the sensitisation phase, an immune response to the allergen is established at the first encounter with the allergen. Dendritic cells present the allergen to undifferentiated T-helper cells. At this stage, the presence of IL-4 is critical to drive the differentiation into allergen specific Th2 cells.[124] The initial source of this cytokine is not defined, but several cells of the innate immune system have been shown to produce IL-4, such as mast cells and basophils. Allergen specific Th2 cells stimulate B cells to produce allergen specific IgE or IgG1 antibodies. The establishment of an allergen specific T cell and B cell response to the allergen is critical for the further development of the disease as these cells establish a memory response that can get activated after a second encounter with the allergen.

Allergen specific IgE binds to FcεRI, the high affinity receptor for IgE on mast cells and basophils.[103] Mast cells are now not only sensitised for activation upon renewed contact with the allergen, but IgE binding without antigen contact already induces cytokine production and upregulation of FcεRI on mast cells, thereby decreasing the threshold for activation.[134] This effect might be counterbalanced by allergen specific IgG1, that binds to the inhibiting FcγRIIB-IgG receptor.[135] Crosslinking of FcγRIIB and FcεRI by allergens inhibits degranulation of mast cells, and a balance of activating and inhibiting signals by Fc-receptors might determine threshold levels for activation.[136, 137]

Crosslinking of IgE/ FcεRI complexes on basophils and mast cells after allergen contact induces the release of histamine, leukotrienes, and prostaglandins. These inflammatory mediators are increasing vascular permeability and smooth muscle cell contraction [134] and are responsible for most of the immediate symptoms of allergic reactions, such as rhinoconjunctivitis, urticaria, asthma and anaphylaxis, which can occur within minutes after allergen exposure.[101]

Furthermore, expression of cytokines by mast cells and basophils, such as IL-4, IL-5 and IL-13 recruits Th2 cells and eosinophils and is essential for the late phase of allergic reactions and subsequently chronic inflammation. The establishment of the late and chronic phase is also supported by histamine as it is a chemoattractant for mast cells and eosinophils and increased vascular permeability enables the influx of inflammatory cells at the site of the allergic reaction.[138]

Allergic asthma is a chronic inflammatory airway disease and a typical Th2 type immune response is observed in mouse models. Many studies used a model of ovalbumin (OVA) induced allergic airway inflammation to study underlying immunological mechanisms.[139] To induce a Th2 type immune response in mice, the allergens have to be administrated together with an adjuvant, such as aluminium hydroxide (alum) or cholera toxin. The mice are normally systemically sensitised by repeated i.p. injection of allergen and

adjuvant, to induce a Th2 type memory response to the allergen. To induce allergic reactions, mice are challenged by nasal administration of the allergen without adjuvant. The main pathological characteristics of the allergic airway disease in these models are development of hyperresponsiveness of airway smooth muscle cells to unspecific stimulation, hyperplasia of mucus producing goblet cells in the airway epithelium, airway eosinophilia and peribronchial and perivascular inflammation in the lungs.[139]

The immediate reactions of allergies are dependent on IgE mediated release of inflammatory mediators. However, the late and chronic phases are less well understood. Complex interactions of immune cells, cytokines and resident cells cause the development of disease symptoms and Th2 cytokines play a central role in these processes.

For the development of Th2 cells, IL-4 is critical. IL-4 deficient mice fail to develop a Th2 type cytokine response [123] and airway hyperresponsiveness, eosinophilia and allergen-specific IgE production is impaired when IL-4 is neutralised during the sensitisation phase.[124, 140] In the effector phase, IL-4 seems not to be critical anymore for the development of disease symptoms as IL-4 neutralisation during airway allergen challenge did not abolish asthmatic symptoms.[124, 140]

IL-13 shares some structural characteristics with IL-4 and has overlapping functions. Both are predominantly produced by Th2 cells, mast cells, basophils and eosinophils [88] and IL-13 promotes development of alternatively activated macrophages, a cell type that has been associated with Th2 type immune responses and allergies.[141] IL-13 might also have an indirect effect of development of Th2 cells and IgE response in mice.[88] Murine T and B cells do not express IL-13 receptors, but IL-13 deficient mice showed impaired Th2 cell development and IgE levels.[142, 143]
Despite some similarities, IL-13 has distinct functions during the development of allergic asthma. The development of AHR, airway eosinophilia and goblet cell hyperplasia, main features of allergic asthma, were induced by IL-13 independently of IL-4.[84, 86] In mouse models, selective blocking of

endogenous IL-13 reduced these symptoms, whereas administration of recombinant IL-13 alone was sufficient for induction. Overexpression of IL-13 in lung epithelium induced mucus production, AHR and airway eosinophilia without allergen challenge.[144]

From *in vitro* studies, it has been suggested that IL-13 induces AHR by a direct effect on contraction of airway smooth muscle cells.[145] Goblet cell hyperplasia and mucus production are dependent on IL-13 [84, 86] and its receptor IL-13Rα1.[109] Mucus production might be controlled by upregulation of $GABA_A$ receptors in airway epithelial cells in response to IL-13, as both have been shown to be essential for mucus overproduction.[146] In conclusion, IL-13 seems to be critically involved in the development of typical symptoms of allergic asthma, whereas IL-4 is necessary for the initial development of an allergen specific Th2 type immune response. However, mice showed goblet cell hyperplasia and AHR after inhalation of recombinant IL-4 in the absence of IL-13 suggesting a common signalling pathway for IL-4 and IL-13 and a degree of redundancy between the two cytokines.[147]

Other cytokines beside IL-4 and IL-13 are important contributors to the development of allergic diseases. IL-5 is a differentiation, migration and activation factor for eosinophils [82] and eosinophilia is a characteristic of Th2 type immune responses. IL-5 deficient mice have impaired numbers of eosinophils in the periphery and the lung after allergen challenge. [148, 149] Overexpression of IL-5 in the lung epithelium induced airway eosinophilia even in the absence of allergen challenge.[150] However, in IL-5 deficient mice, eosinophil numbers are highly reduced in the bronchoalveolar space but are still present in the perivascular and peribronchial inflammatory cell infiltrate.[151] These data suggest a critical role for IL-5 in the development of eosinophilic airway inflammation, but further signals seem to be necessary of the regulation of eosinophilic inflammation in allergic asthma. Eotaxin is a chemokine of the C-C family with specific chemotactic properties for eosinophils, which is critically involved in the development of allergic tissue eosinophilia.[152] In mice deficient IL-5 and eotaxin, peribronchial and perivascular eosinophilia was reduced, suggesting cooperative effects

between IL-5 and eotaxin regulate eosinophil recruitment in the allergic lung.[151]

Despite the fact that eosinophils have been associated with allergic asthma for a long time, the role of these cells in the development of the disease is controversial. Eosinophils are able to secrete inflammatory mediators, such as leukotrienes, EPO, MBP or ECP but have also been identified as producers of the Th2 cytokines IL-4 and IL-13.[102] These properties make a prominent role of these cells in the development of allergic asthma plausible and numbers of eosinophils have been correlated with disease severity in humans. However, clinical trials in humans with anti-IL-5 monoclonal antibodies did reduce eosinophil numbers in asthmatic patients and did not improve disease conditions considerably.[83] Experimental models in eosinophil or IL-5 deficient mice gave conflicting results. Neutralisation of IL-5 by monoclonal antibodies reduced eosinophil numbers dramatically, but had no effect on airway hyperresponsiveness,[140] whereas IL-5 deficient mice where protected from the development of allergic airway symptoms.[149, 151] Two different transgenic mouse strains with a deficiency in eosinophil generation showed conflicting results. ΔdblGATA mice developed AHR and goblet cell hyperplasia [153] whereas *PHIL* mice were completely protected from AHR, and partially protected from goblet cell hyperplasia.[154] An explanation for the differences in these transgenic mouse strains might be their different genetic background, as ΔdblGATA mice were bred from Balb/c, and *PHIL* mice from C57/Bl6 mice.[155] Balb/c mice develop stronger Th2 type immune responses and AHR in models of allergic airway disease than the C57/Bl6 strain, and contributions eosinophils to Th2 type immunity might be less dominant in Balb/c mice.

Further cytokines are involved in development of allergic asthma, probably by enhancing Th2 type immune responses. Pulmonary overexpression of IL-9 [156] and IL-25 (IL-17E) [157] increased numbers of Th2 cells and resulted in enhanced mucus production, eosinophilia and AHR.

A dual role for IL-17 has been demonstrated in allergic asthma and a role in regulating disease progression has been suggested. Whereas signals of the

IL-17-receptor are required for allergic sensitisation, expression of IL-17 in the effector phase of allergic asthma reduces allergic symptoms by inhibiting dendritic cell functions and expression of IL-4 and IL-5.[158]

1.2.6 IL-4 and IL-13 receptors

IL-4 and IL-13 are playing a central role in allergic sensitisation and development of allergies symptoms and show overlapping functions. This is at least partially due to signalling through receptor complexes which with the IL-4Rα chain as a common component.[88]

The IL-4Rα forms a least two functional receptor complexes. Type I IL-4 receptor consists of the IL-4Rα and the common γ-chain (γc) of cytokine receptors, which is shared with other cytokine receptors, such as IL-2, IL-7, IL-9, IL-15 and IL-21. The type II receptor consists of the IL-4Rα and the IL-13Rα1 chain, a low affinity receptor specific for IL-13. IL-4 can signal through type I and type II receptors whereas IL-13 only binds to type 2 receptors.[88] For both receptor complexes, intracellular signal transduction is mediated by signal transducer and activator of transcription 6 (STAT 6) or insulin like receptor substrate (IRS) family proteins.[88] IL-4 and IL-13 have different affinities to the shared type II receptor complex, which results in different intracellular signal quality for IL-4 and IL-13.[159] In contrast to the type I receptor, the type II IL-4 receptor is not expressed on T cells, explaining the dependency of Th2 cell differentiation on IL-4 but not IL-13 signals. The difference between the responsiveness of human and mouse B cells to IL-13 for IgE class switch is also due to different expression of the type II receptor.

With the exception of lymphocytes, type I and type II IL-4 receptors are widely expressed throughout different tissue and cell types. IL-13Rα2, a high affinity receptor for IL-13, exists in membrane bound and soluble forms. It has been described as a decoy receptor as it lacks intracellular signalling functions.

Furthermore, it inhibits IL-13 dependent symptoms in experimental asthma and might provide a negative feedback mechanism in allergic diseases.[160]

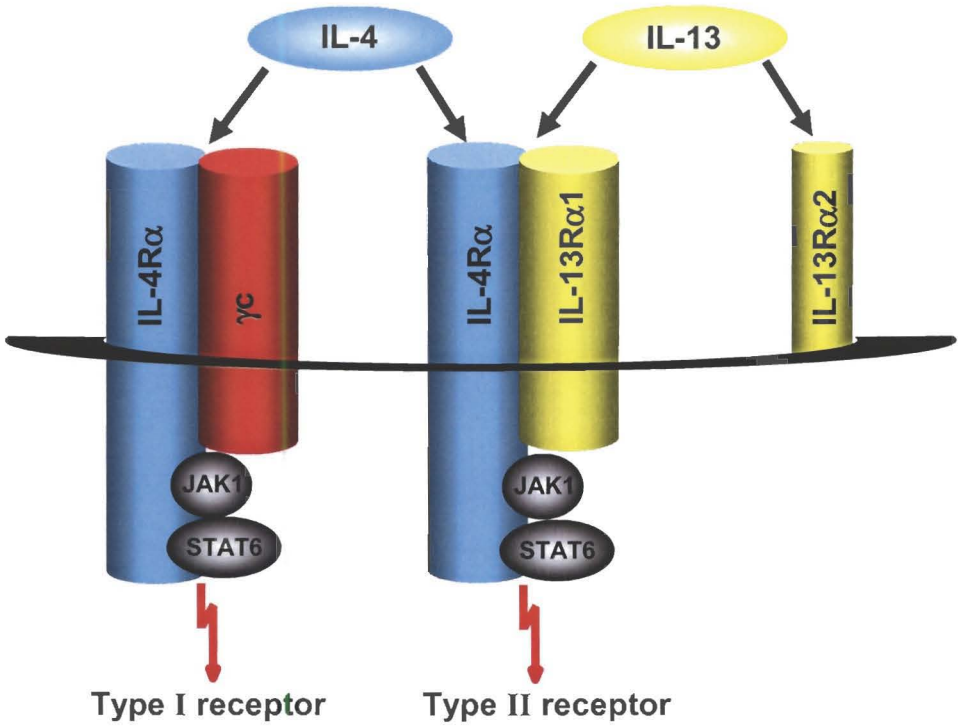

Figure 5: IL-4 and IL-13 receptor complexes. IL-4 interacts with type I and II receptor complexes. IL-13 interacts with the type II receptor complex and with IL-13Rα2. Type I and type II receptors signal through the JAK1/STAT6 pathway.

1.2.7 IL-4Rα in allergies and asthma

Many cells, directly involved in the pathology of allergic asthma are expressing functional IL-4 and IL-13 receptors.[88] The importance of these cytokines in disease development gives the IL-4Rα chain a central role in the induction of allergic symptoms. Blocking of IL-4Rα impaired the development of AHR and airway eosinophilia in experimental allergic asthma [161, 162] and IL-4Rα deficient mice do not develop a Th2 type cytokine response and are

protected from the development of goblet cell hyperplasia, eosinophilic airway inflammation and allergen specific IgE production.[86] Instead, IL-4Rα deficient animals show antigen specific Th1 type cytokine and antibody responses and airway inflammation, characterised by high neutrophils numbers. However, IL-13 and IL-5 dependent development of AHR and specific IgE in IL-4Rα deficient mice has been demonstrated.[163-165]

IL-4Rα signals contribute to the development of Th2 cells, but the contribution of Th2 cells in allergy development needs to be defined. These cells are associated with allergic asthma and their production of Th2 cytokines is thought to be critical for disease development. Several studies are supporting this concept as $CD4^+$ T cells are necessary for development of airway eosinophilia and AHR [124] and allergen specific Th2 cells are able to induce allergic airway symptoms.[166, 167] However, a recent study suggested that Th2 cell derived IL-4 and IL-13 may not be necessary for the development of allergic symptoms and that the main source of IL-4 and IL-13 in allergy might be non-eosinophil cells of the innate immune system.[168]

Cells of the innate immune system, such as basophils and mast cells, are critically involved in the disease pathology of allergic asthma and are important producers of Th2 cytokines.[103] These cells express IL-4Rα [88] and IL-4 and IL-13 responsiveness may contribute to the development of a Th 2 type immune response.[169, 170]

Development of so called alternatively activated macrophages (AAM) is induced by IL-4 and IL-13 signals through IL-4Rα.[141] AAM are found in high numbers in experimental and human asthma and produce several allergy associated molecules, such as arginase and Ym1.[141] Some studies suggested a role of AAM in eosinophil recruitment [171] and contributions to tissue remodelling in the chronic phase of allergic asthma.[141] However, a functional role in development of allergic asthma has not been demonstrated yet.

Airway hyperresponsiveness is caused by contraction of airway smooth muscle cells (ASMC) to a specific or unspecific stimulus and it is apparent that these cells play an important role in the onset of asthma. Several *in vitro* studies, showed clear evidence for a direct involvement of the IL-4Rα on smooth muscle cells in this process.[145] Stimulation of cultured ASMC with IL-4 or IL-13 increased hypercontractility in a STAT 6 dependent manner.[172-174] Furthermore, *in vitro* data suggested that IL-4Rα signalling on ASMC contributes to airway inflammation by release of inflammatory cytokines, such as eotaxin.[175, 176] It is currently believed that IL-4Rα responses of ASMC contribute significantly to the onset of allergic asthma, but these effects still need to be demonstrated *in vivo*.

Allergic asthma is characterised by a complex system of different overlapping symptoms, involving a large number of IL-4Rα expressing cell types. Although the general importance of the IL-4Rα in disease development is well established, its cell type specific functions often remain elusive. For many cells involved in disease development the role of IL-Rα is controversial or not understood and several open questions remain.

The development of the *loxP/Cre* system for cell specific gene targeting enabled development of mouse models that allow investigation of gene defects on a cellular level.[177] In transgenic mice, Cre recombinase expression under control of a cell type specific promotor induces homologous recombination between two *loxP* sites that flank essential exons of a gene of interest, leading to the disruption of the gene.[178] This technology was used to generate several transgenic mouse strains with cell type specific disruptions of the *il4ra* gene on a Balb/c genetic background.[179-183] Exons seven through nine of the *il4ra* gene are excised in target cells, whereas all other cell types carry a functional *il4ra* gene with a silent mutation, due to the insertion of *loxP* sites into intron regions (IL-4Rα$^{lox/lox}$ mice). The efficiency of gene deletion was enhanced by generation of hemizygous mice carrying one *loxp il4ra* allele and one constitutively disrupted allele (IL-4Rα$^{-/lox}$ mice).[180-182] Previous studies

demonstrated a Balb/c phenotype for hemizygous IL-4Rα$^{-/lox}$ mice and these mice were used as wild type control animals in this study. [180, 183]

Mice with cell type specific disruption of the *il4ra* gene allow the investigation of cell type specific roles of the IL-4Rα in disease development. This approach was successfully used to demonstrate that IL-4Rα signalling in clara cells, goblet cell precursors in the airway epithelium, is required for allergen-induced mucus production in experimental asthma.[181] Disruption of the *il4ra* gene in clara cells had no effect on other allergic symptoms.

This was the first study that demonstrated that IL-4Rα signalling in a single cell type was sufficient for the development of a major symptom of allergic airway disease. Generation of transgenic mice with cell type specific disruptions in the *il4ra* gene in macrophages [180] and smooth muscle cells [183] are an excellent tool for defining a role for specific contributions of IL-4Rα signals in these cells in the onset of allergic airway disease.

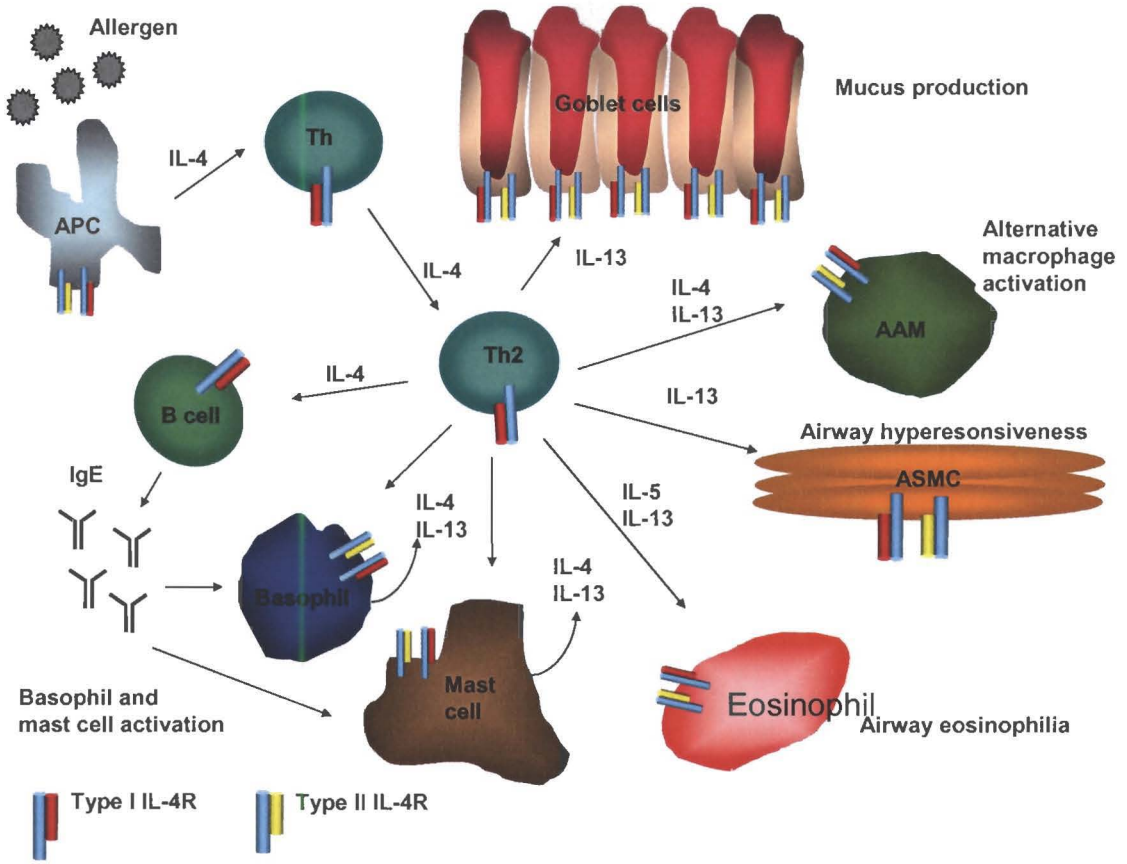

Figure 6. Potential roles for IL-4Rα in the development of allergic asthma. IL-4Rα is expressed on various cell types associated with the early phase of allergic asthma. Cell specific contributions of IL-4Rα signals to the disease pathology remain to be defined. Th, T helper cell; AAM, alternatively activated macrophage; ASMC, airway smooth muscle cell. Illustrated according to the above literature.

1.3 Objectives of this project

Aim of the present study was to investigate influences of different routes of sensitisation on the development of allergic airway disease with focus on the role of IL-4Rα in mouse models.

Mouse models of allergic diseases have demonstrated that the route of sensitisation is influencing allergic symptoms and underlying immune responses.[59, 92] The fish parasite *Anisakis* can induce allergic reactions in humans and accidental infection has been considered as an important way of sensitisation.[56] Furthermore, studies from our laboratory and others have shown that exposure to aerosolised allergens from *Anisakis pegreffii* increases the risk of developing allergic airway symptoms.[59, 60] Currently it is not clear if different exposure routes to *Anisakis* affect the development of allergic asthma and if they have an influence on the immune response. To address these questions, the present study investigated immune responses and allergic symptoms after *Anisakis* live infection and nasal allergen sensitisation in a mouse model of allergic airway disease.
A central role in the development of allergic diseases plays IL-4Rα. Contribution of IL-4Rα after live infection or nasal sensitisation with *Anisakis* was investigated by comparing IL-4Rα deficient mice with wild type Balb/c mice.

IL-4Rα is expressed on a variety of cells involved in allergic asthma but its cell specific contributions to disease development remain to be defined. Contraction of airway smooth muscle cells (ASMC) plays a central role in asthma pathology. From *in vitro* studies, it is currently believed that signals of IL-4Rα on ASMC contribute to airway hyperresponsiveness and airway inflammation.[145] To test this hypothesis *in vivo*, $SMC^{cre}IL\text{-}4Ra^{-/lox}$ mice, [183] which are deficient in ASMC IL-4Rα, were investigated in a mouse model of ovalbumin induced allergic airway disease.

AAM are a feature of allergic asthma in human studies and mouse models [141] but no functional role in disease development could be defined so far. LysM^{cre}IL-4R$\alpha^{-/lox}$ mice have a cell type specific disruption if the *il4ra* gene in macrophages and neutrophils and show impaired AAM development.[180] These mice were used in a model of ovalbumin induced allergic airway disease in order to define a role for AAM in the development of allergic symptoms.

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Chapter 2

The Route of Sensitization to *Anisakis pegreffii* influences the Outcome of Experimental Allergic Asthma

2 The Route of Sensitization to *Anisakis pegreffii* influences the Outcome of Experimental Allergic Asthma

2.1 Summary

Background

The fish parasite *Anisakis pegreffii* is able to cause allergies after accidental infection of humans and in the occupational environment. The present study examines differences in the immune response and development of allergic asthma that are due to different routes of sensitisation to *Anisakis* allergens.

Methods

Wild type and IL-4Rα deficient mice were either sensitized by infection with live larvae of *Anisakis pegreffii* or by intranasal administration of *Anisakis* extract. Allergic airway responses were induced by intranasal challenge with *Anisakis pegreffii* extract and mice were analysed for airway hyperresponsiveness, airway inflammation, allergen specific antibody production, Th2 type cytokine responses and lung pathology.

Results

Mice developed allergic asthma after infection with live *Anisakis* larvae. Airway hyperresponsiveness was independent of IL-4Rα but expression of this receptor was crucial for eosinophilic airway inflammation and goblet cell hyperplasia.

Nasal sensitisation with *Anisakis* protein extract induced typical symptoms of allergic asthma in Balb/c mice. Airway hyperresponsiveness, eosinophilic airway inflammation and goblet cell hyperplasia were dependent on IL-4Rα but independent of a systemic allergen specific antibody response.

Conclusion

This study showed that the route of sensitisation influences the outcome of *Anisakis pegreffii* induced allergic asthma and its underlying immune response. Sensitisation by infection with live larvae and inhalation of proteins can be important routes of sensitisation of humans to *Anisakis* and different ways of sensitisation may explain differences in the manifestation of the disease.

2.2 Introduction

Anisakis spp. are nematode parasites of marine mammals with a worldwide distribution and a complex live cycle which includes several intermediate hosts. L3 larvae of *Anisakis pegreffii* are frequently found in commercially important sea fish designated for human consumption and infection levels in South African fish populations reach 95-100%.[1] Most parasites live in the visceral cavity of infected fish but occasionally a few individuals migrate into the muscle of the host. The prevalence of *Anisakis* worms in fish fillets varies considerably depending on the fish species[2]. Eating contaminated fish can cause health problems in humans as infection with L3 larvae may occur after consumption of raw or undercooked fish infested with the parasite. Common sources of infection are raw, smoked or pickled fish and most cases are reported from countries like Japan and Spain where these dishes are frequently consumed.[3-5] After infection the larva invades the gastric or intestinal mucosa by secreting proteases,[6] causing painful tissue damage and inflammatory reactions. The disease caused by infection with live larvae is called gastric or intestinal anisakiasis. Symptoms such as nausea, abdominal pain and vomiting may only appear within 12h after ingestion which is why they are often not related to fish consumption.[7] Acute anisakiasis often remains undiagnosed as clinical features of infection are similar to gastric ulcer or appendicitis.[8] Endoscopic examination is the best way of diagnosis [9] and symptoms disappear after removal of the larvae.[7] The health risk of *Anisakis* infection has been recognised and the European Union and United States Food and Drugs Administration demand freezing and visual controls of

uncooked fish designated for consumption in order to prevent development of acute or chronic anisakiasis.[3]

Cases of hypersensitivity reactions to Anisakis, so called gastroallergic anisakiasis have been reported in patients infected with *Anisakis*.[10, 11] Symptoms range from urticaria and angioedema[7] to live-threatening anaphylaxis.[11, 12] Hypersensitivity symptoms are mediated by *Anisakis*-specific IgE as opposed to symptoms of gastric anisakiasis and usually appear between 1-2h after fish consumption.[3] Allergic reactions to *Anisakis* are thought to be a memory response to a previous infection after a second encounter with *Anisakis* antigens.[13] It is still controversial whether consumption of fish contaminated with *Anisakis* proteins is sufficient for sensitisation and development of allergic reactions or if this reactions require the presence of live larvae.[3] Sensitised patients were symptom free after a frozen fish diet or oral challenges with *Anisakis* protein preparations[14-16] and it was concluded that the live larvae is necessary for the development of hypersensitivity reactions. Nevertheless, allergic reactions to *Anisakis* have also been reported after consumption of well cooked or previously frozen fish indicating that proteins of the worm might be sufficient for sensitisation and hypersensitivity reactions.[17-21] Several allergens of *Anisakis* are heat stable and pepsin resistant [22, 23] and cooking might therefore not destroy their allergenic capacities.[24] In countries with high fish consumption a large proportion of cases of food allergies and anaphylactic reactions can be due to *Anisakis*. In Spain, *Anisakis* is one of the most common hidden food allergens.[25]

Immunological mechanisms behind human anisakiasis or *Anisakis* allergies remain to be defined. Penetration of gastric or intestinal tissues evokes an inflammatory response with eosinophilic infiltrates of the gastrointestinal wall around the parasite and blood eosinophilia.[26-28] *Anisakis* protein extract has been shown to be chemotactic for eosinophils but not neutrophils.[29] Eosinophils and lymphocytes are present at the site of inflammation caused by *Anisakis* infection and expression of IL-4 and IL-5 but no detectable IFN-γ was confirmed by PCR analysis. Production of IL-5 but not IFN-γ and

proliferation of blood lymphocytes from patients was stimulated by *Anisakis* antigen.[8] These results show characteristic features of a Th2 type immune response as typically seen in nematode infections and allergic reactions. In an experimental mouse model, infection with *Anisakis* induced a IL-4 dependent Th2 type immune response with increased levels of specific IgE and IgG1[30] which is comparable to the situation in human patients.[8, 31] Subsequent oral challenge of sensitised mice with *Anisakis* extract induced mast cell degranulation, histamine release and symptoms of food allergy.

Whereas urticaria, angioedema and anaphylaxis are the most common symptoms of *Anisakis* allergy several studies also have reported respiratory manifestations of the disease. In food allergic patients a correlation between asthmatic symptoms and *Anisakis* sensitisation was found in a Spanish study.[32] Respiratory symptoms are common features of food allergies [33, 34] and patients with sensitisation to *Anisakis* might be at risk of developing allergic asthma after consumption of contaminated fish. A recent study from our group supported this possibility. In an experimental mouse model for *Anisakis* induced food allergy it was shown that mice develop symptoms of allergic airway disease after infection and intragastric challenge with *Anisakis*.[30] Cases of occupational conjunctivitis [35] and high prevalence of sensitisation in fishermen and fish mongers [36] suggested that *Anisakis* is also an airborne allergen causing symptoms after inhalation. A Spanish study reported the first case of occupational asthma caused by *Anisakis* in workers exposed to contaminated fish meal or fish.[37] A recent study from our group found a correlation between fish consumption, *Anisakis* sensitisation and airway hyperresponsiveness in fish factory workers in South Africa[30,38-40]. Other case reports showed occupational asthma due to *Anisakis* after handling frozen fish,[41] fish meal[42] or exposure to fumes from cooked fish.[43] Whereas live infection with *Anisakis* seems to be the most important way for the development of food allergies, sensitization by inhalation of *Anisakis* proteins might be important in occupational allergies.

Mouse models are frequently used to study underlying mechanisms of allergic diseases. Immune responses that finally lead to the development of allergic diseases can be investigated in mice under controlled experimental

conditions. Mouse models of allergic asthma show similarities to the human disease such as airway hyperresponsiveness, goblet cell hyperplasia and airway inflammation.[44,45] The use of genetically modified mice allows investigation of the role of specific genes in disease development.

In sensitised mice the development of allergic asthma after oral challenge with *Anisakis* allergens was dependent on IL-4 and IL-13 [30] but immunological mechanisms of *Anisakis* induced allergic asthma after inhalation of the allergens have not been investigated yet. The aim of this study was to compare the impact of two different routes of sensitisation to *Anisakis pegreffii* on the outcome of experimental allergic asthma. Mice were either infected intraperitoneally with live *Anisakis* larvae or exposed to *Anisakis* extract via the nose. The effect on allergic sensitisation was investigated by comparing specific antibody and cytokine responses. Allergic asthma was induced by intranasal challenge with *Anisakis* extract and airway inflammation, airway hyperresponsiveness, lung pathology and mucus production was compared between the two sensitisation groups. From mouse models of allergic diseases it is known that the development of allergic symptoms is dependent on the cytokines IL-4 and IL-13 and their common receptor IL-4Rα,[46, 47] therefore the development of allergic asthma in response to *Anisakis* was compared between IL-4Rα deficient and wild type Balb/c mice.

2.3 Materials and Methods

2.3.1 Mice

The experiments were performed with 6-8 weeks old female wild type Balb/c mice or IL-4Rα$^{-/-}$ mice on a Balb/c genetic background.[48] Animals were housed in the animal unit of the University of Cape Town under specific pathogen free barrier conditions using individually ventilated cages. All experiments were approved by the Research Animal Ethics Committee of the University of Cape Town.

2.3.2 Anisakis larvae

L3 stage larvae of *Anisakis pegreffii* were collected from the viscera of parasitized snoek (*Thyrsites atun*) and washed in 4% acetic acid and phosphate-buffered saline (PBS). Larvae were frozen at -80°C for protein extract or stored live at 4°C in Iscove's Modified Dulbecco's medium (IMDM) containing 100μg/ml penicillin and 100μg/ml streptomycin (Gibco, UK). Before use in live infections, larvae were re-washed in sterile PBS.

2.3.3 *Anisakis* extract

Frozen *Anisakis* larvae were homogenized in PBS and then sonicated with a MicrosonTM ultrasonic cell disrupter (Misonix, USA). The extract was centrifuged for 15min at 20000rcf and filter-sterilized through a 0.20μm filter (Sartorius, Germany). Protein concentration was measured by BCA test (Pierce, USA) according to manufacturer's instructions. *Anisakis* extract was kept on ice during the extraction process and stored at -80°C.

2.3.4 *Anisakis* live infection and intranasal challenge

Mice were anaesthetized with 200µl of ketamine (Centaur Labs, South Africa)/xylazine (Bayer, South Africa) anaesthetic, and then injected intraperitoneal with two larvae in 1ml PBS at day 0 and day 21 using a 12 gauge needle (Identipet, South Africa). On days 42, 43 and 44 mice were anaesthetised with 200µl Ketamine (Centaur Labs, South Africa)/ Xylazine (Bayer, South Africa) and challenged with 0.1mg *Anisakis* protein extract in 50µl PBS by intranasal administration. PBS control groups were treated identically except *Anisakis* larvae were missing in the solutions. Mice were killed and analysed on day 46.

2.3.5 Nasal allergen sensitisation and intranasal challenge

Mice were anaesthetized with 200µl Ketamine (Centaur Labs, South Africa)/ Xylazine (Bayer, South Africa) and sensitised on day 0, 7 and 14 with 50µg *Anisakis* protein extract in 10µl PBS/ 1.3% aluminium hydroxide (Sigma) by intranasal administration. On days 21, 22 and 23 mice were anaesthetised with 200µl Ketamine (Centaur Labs, South Africa)/ Xylazine (Bayer, South Africa) and challenged with 0.1mg *Anisakis* extract in 50µl PBS by intranasal administration. PBS control groups were treated identically except *Anisakis* was missing in the solutions. Mice were killed and analysed on day 24 or day 25 if airway if airway hyperresponsiveness was measured.

2.3.6 Airway Hyperresponsiveness

Responsiveness to β-methacholine (Sigma) was assessed in conscious unrestrained mice using a whole body plethysmograph (emka Technologies, France) and analysed with iox2 software (emka Technologies, France). This system measures pressure changes induced by the breathing of the mice and

computes enhanced pause (Penh) values, a dimensionless parameter calculated by the formula

Penh= (expiratory time / relaxation time-1)
x (peak expiratory flow / peak inspiratory flow) [49]

Increasing duration of the late phase of expiration relative to its early phase and increasing pressure change during expiration relative to the pressure change during inspiration results in increasing Penh values.[50] It has been shown previously that changes in Penh values correlate with changes in airway resistance in the Balb/c mouse strain under similar conditions to those used here.[49, 51, 52] Individual mice were placed in plethysmograph chambers and baseline readings were measured for 5min before each exposure to increasing doses of aerosolised methacholine (0, 10, 20, 40 mg/ml methacholine in PBS). The responses were measured for 15 min after each nebulisation. Results are computed as average of first 5 min minus average of baseline reading.

2.3.7 Serum antibodies

Blood samples were taken by tail vein bleeding or directly from mice after killing and collected in plasma separator tubes (Microtainer™ SST, BD, USA). Samples were centrifuged for 20min at 6000rcf and stored at -80°C. *Anisakis* specific antibodies were measured in blood serum by endpoint titration ELISA. For antigen specific antibody-ELISA 96 well plates (Maxisorp, Nunc, Denmark) were coated overnight with 50µl *Anisakis* extract in PBS (5µg/ml for IgG1, IgG2a, IgG2b or 100µg/ml for IgE). For total IgE ELISA plates were coated with anti-mouse IgE antibody (clone 84.1C, 1/1000 dilution). Plates were blocked for 1h at 37°C with 200µl 2% fat free milk powder in PBS. Serial dilutions of serum samples in 50µl PBS/ 0.1% BSA were added to the wells and plates were incubated over night at 4 °C. Purified recombinant mouse IgE (BD, USA) was used as a standard for total IgE ELISA, at a starting concentration of 1µg/ml. 50µl alkaline phosphatase conjugated goat anti-

mouse isotype specific antibodies (Southern Biotechnology, USA) at a 1/1000 dilution in PBS/ 0.1% BSA were added to the wells and the plates were incubated for 3h at 37°C. Subsequently plates were incubated with 50µl p-nitrophenylphosphate (Fluka, Switzerland) and colour reaction was stopped with 50µl 1M NaOH. Absorption was measured at 405nm with 492nm as reference wave length using a VersaMax plate reader (Molecular devices, USA). Plates were washed 4x with 200µl per well after each incubation step.

2.3.8 Western Blot Analysis

Anisakis extract was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A 10% separating gel (14.5x11cm) was prepared first and left to polymerize for 30 minutes. The stacking gel was poured onto the separating gel and a 1-well comb (+1 reference well for molecular weight marker) was added. 200µg protein in 160µl TBS and 40µl 5xSDS-sample buffer was boiled for 5 min before being loaded onto the gel. 6µl Precision Plus Dual Color molecular weight marker (Bio-Rad, USA) was loaded into the reference well. Proteins were separated at 150V over a length of 8cm. After SDS-PAGE proteins were transferred onto a nitrocellulose membrane (Amersham, UK) for 1h at 400mA. The membrane was blocked for 1h at RT in TBS/0.1% Tween/5% fat free milk powder before it was placed in a 21 lanes Western Blot Screening Device. 200µl serum samples were loaded per lane in a 1/100 dilution in TBS and incubated for 3h (for IgG) or overnight (for IgE). Afterwards the membrane was washed 3x in TBS/0.1% Tween and incubated with horseradish peroxidase (HRP) labelled rat anti-mouse IgE (ε-chain specific, Southern Biotechnology, USA) for 3h. After 3x washing in TBS/ 0.1% Tween the Western blot was developed with 2ml ECL solution (Amersham, UK). Antibody binding was visualized by using x-ray films (Fuji, Japan).

2.3.9 Bronchoalveolar Lavage

Mice were killed by CO_2 asphyxiation and lungs were washed once with 1ml PBS through an 18G IV catheter (Braun, Germany). Recovered bronchoalveolar lavage (BAL) fluid of individual mice was centrifuged for 10min at 400rcf and the cell pellet was resuspended in 0.5ml DMEM after. Total cell numbers in the BAL fluid were counted in trypan blue (SIGMA, Germany) stained aliquots using a haemocytometer. A maximum of $2x10^5$ cells in 200µl was centrifuged on a microscope slide at 800rpm for 5 min using a Cytospin centrifuge (Shandon, UK). The slides were air-dried and stained with a Rapidiff Stain Set (Clinical Diagnostics CC, South Africa). Differential cell counts for eosinophils, neutrophils, lymphocytes and macrophages were made at 400x magnification and at least 100 cells were counted per slide.

2.3.10 *In vitro* restimulation of lymphocytes

Lymphocytes were purified from lung draining lymph nodes for *in vitro* restimulation experiments. Lymph nodes were removed from the mice and pooled in DMEM/ 2% FCS for each experimental group. Lymph nodes were pressed through a 70µm cell strainer (BD, USA) and the single cell suspension was washed once with 10ml DMEM/ 2% FCS. The pellet was resuspended in 1ml of ice-cold red cell lysis buffer for 2-5 minutes on ice and cell lysis was stopped by adding 10ml DMEM/ 2% FCS. The cells were centrifuged at 300rcf for 5 minutes. $2x\ 10^5$ lymphocytes per well were restimulated in U-bottom 96-well plates (Nunc, Denmark) with 100mg/ml *Anisakis* protein extract in IMDM / 10% FCS or in IMDM / 10% FCS alone at 37°C.

2.3.11 Cytokine ELISA

Cytokine concentrations in the supernatant of *in vitro* restimulated lymphocytes were determined by ELISA. Plates (Maxisorp, Nunc, Denmark) were coated overnight with 50µl anti-IL-4 (clone 11B11), anti-IL-5 (BD, USA), anti-IL13 (R&D Systems, USA) or anti-IFNγ (clone AN18KL6) capture antibodies at 4°C. Plates were blocked for 1h at 37°C with 200µl 2% fat free milk powder in PBS. Samples or serial dilutions of recombinant cytokine standards (BD, USA) in 50µl PBS/ 0.1% BSA were added to the wells and plates were incubated overnight at 4 °C. Biotinylated secondary antibodies were added and plates were incubated for 3h at 37°C followed by horseradish peroxidase (HRP) labelled streptavidin for 1h at 37°C. Subsequently plates were incubated with 50µl TMB Peroxidase Substrate (KPL, USA) and colour reaction was stopped with 50µl 2M H_2SO_4. Absorption was measured at 450nm with 540nm as reference wave length using a VersaMax plate reader (Molecular devices, USA). Plates were washed 4x with 200µl of wash buffer per well after each incubation step.

2.3.12 EPO assay and MMCP-1 ELISA

Lung tissue was homogenized in Tris/ NP-40 buffer and then centrifuged for 20 minutes at 14000rpm for mouse mast cell protease-1 (mMCP-1) ELISA and Eosinophil Peroxidase (EPO) assay. A colorimetric assay for EPO was used to quantify eosinophils in the lung tissue. 50 µl of the substrate solution consisting of 16mM o-phenylenediamine (Sigma, Germany) in 100mM Tris-HCl buffer pH 8.0 containing 0.1% Triton and 0.01% hydrogen peroxide was added to 50µl tissue sample (protein concentration 1mg/ml) and peroxidase standard (Sigma, Germany) in 96 well plates. The reaction was stopped after 5 minutes with 50µl 2M H_2SO_4 and absorption was measured at 490nm. Mouse Mast Cell Protease concentrations were determined by ELISA (Moredum Scientific, UK) according to manufacturer's protocol.

2.3.13 Lung Histology and Immunohistochemistry

Lung tissue was fixed in 4% Formaldehyde in PBS, embedded in paraffin and cut in 5-7μm sections. Sections were stained with Periodic acid-Schiff reagent (PAS) or haematoxylin and eosin (H&E).

2.3.14 Statistical Analysis

Values are given as mean ± SD and significant differences were determined using ANOVA-test (Graphpad Prism™ software). Values $p < 0.05$ were considered significant.

2.4 Results

2.4.1 Antibody responses after infection of mice with *Anisakis* larvae and nasal exposure to *Anisakis* extract

Two new mouse models were used in the present study to investigate the influence of the route of sensitisation on the development of allergic airway disease in response to *Anisakis pegreffii*. In the first model mice were infected twice with live *Anisakis* larvae (Fig 1A) whereas in the second model mice were sensitised by nasal administration of Anisakis extract (Fig. 1B). In both models, mice were challenged after sensitisation intranasally with *Anisakis* protein extract to induce allergic airway responses.

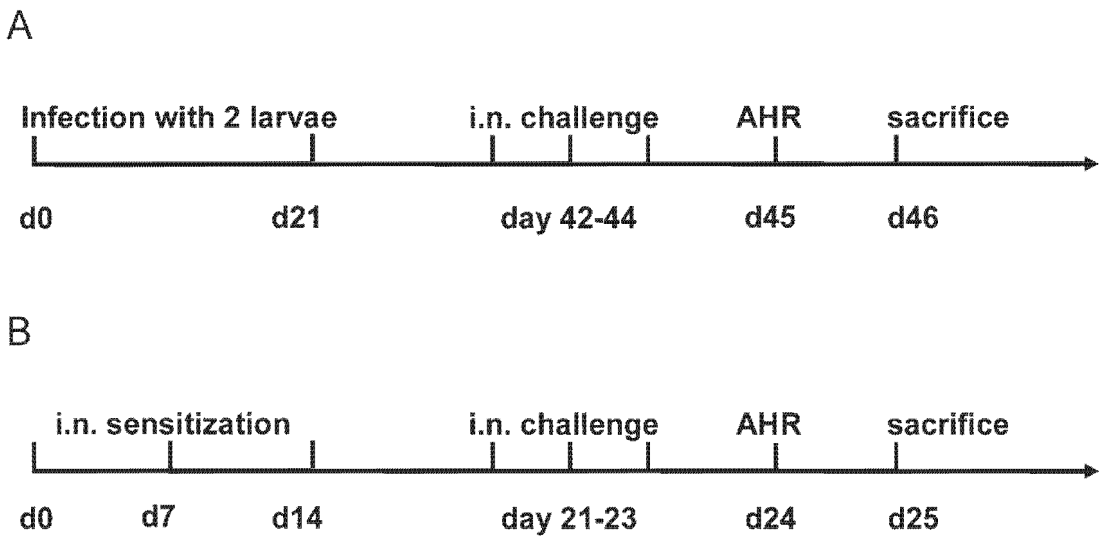

Figure 1. Live infection with *Anisakis* larvae or nasal sensitisation with *Anisakis* extract followed by intranasal challenge with *Anisakis* protein extract. Mice were infected intraperitoneally with 2 live *Anisakis pegreffii* larvae on day 0 and day 21 (A) or sensitised intranasally (i.n.) with 50µg *Anisakis* extract in 10µl PBS/ Alum on day 0, 7 and 14 (B). This was followed by 3 intranasal (i.n.) challenges of anesthetized mice with 0.1mg *Anisakis* extract in 50µl PBS. Airway hyperresponsiveness (AHR) was measured one day before mice were sacificed for analysis. The PBS control group was treated identically except antigens were absent from the solutions.

To compare antibody responses after repeated infection of mice with *Anisakis* larvae, specific antibody levels were determined by ELISA in Balb/c and IL-4Rα$^{-/-}$ mice on days 21 and 45 post infection. Serum levels of *Anisakis* specific IgG1 and IgE, typical Th2 type antibodies, were significantly increased in infected Balb/c mice on day 21 post infection. Reinfection further elevated the serum antibody titres by day 45 (Fig. 2A). In accordance with the specific antibody response, total IgE concentrations were elevated after first infection and further increased after the second infection (Fig. 2B). IL-4Rα$^{-/-}$ mice produced specific IgG2a and IgG2b and to a lesser extent IgG1 and the concentrations of specific IgG were further increased by the second infection with *Anisakis* larvae (Fig. 2A). Specific and polyclonal IgE were not detected in IL-4Rα$^{-/-}$ mice (Fig. 2A).

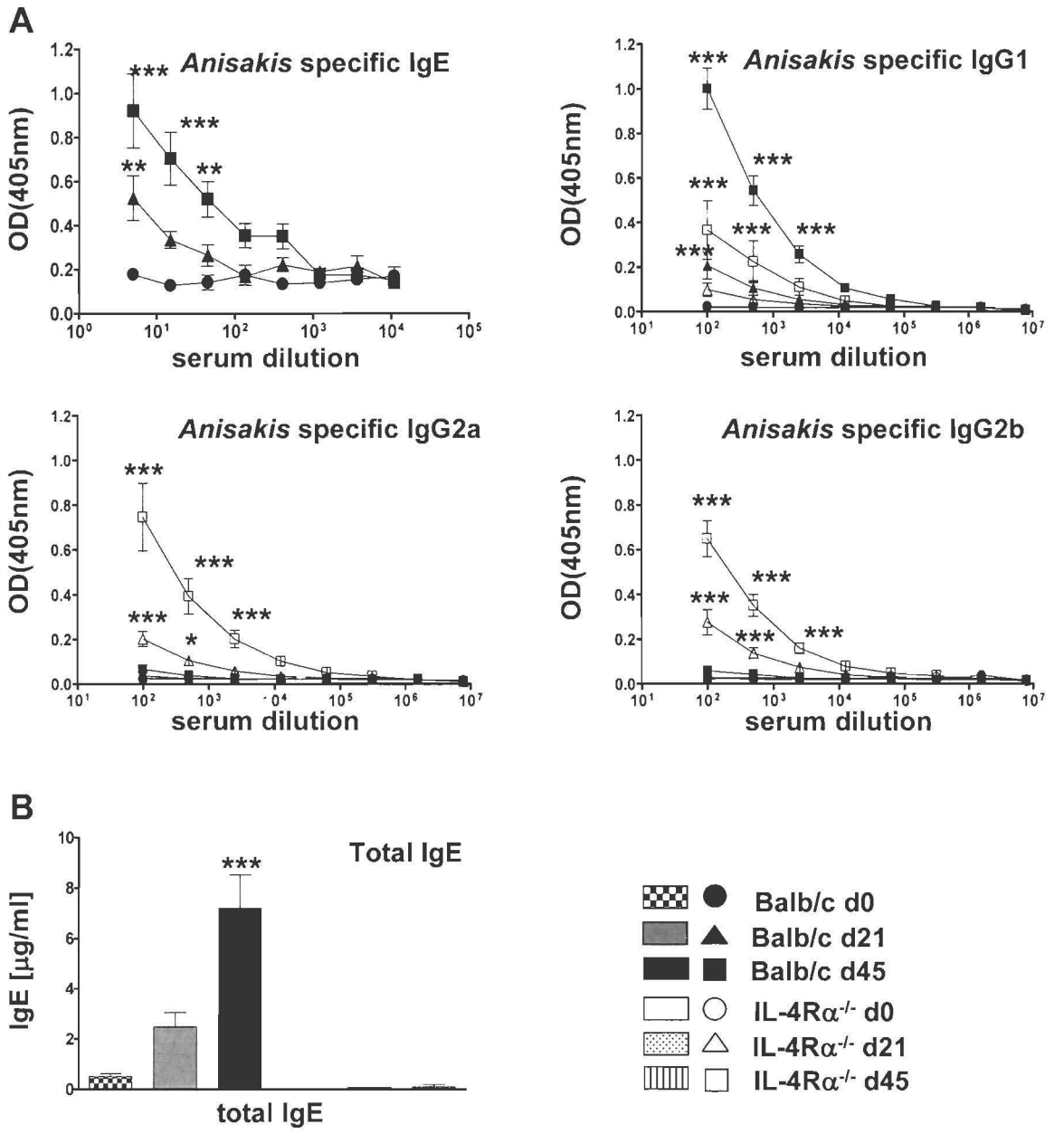

Figure 2. Repeated infection of mice with *Anisakis* larvae induces a strong specific antibody response. Balb/c and IL-4Rα$^{-/-}$ mice were infected with live *Anisakis* larvae on day 0 and day 21. Serum concentrations of antigen specific IgE and IgG (A) and total IgE (B) were determined 3 weeks after each infection. Data represent the pool of two experiments +/-SD (*p<0.05; **p<0.01; ***p<0.001 vs. PBS control group).

In contrast to the high antibody titres after infection with *Anisakis*, repeated nasal sensitisation and challenge only induced a weak specific IgG1 response in Balb/c mice (Fig. 3A). Concentrations of total IgE were not affected by intranasal *Anisakis* sensitization (Fig. 3B). No increase in specific IgG

concentrations was found in IL-4Rα$^{-/-}$ mice (Fig. 3A) and IgE could not be detected (Fig. 3B).

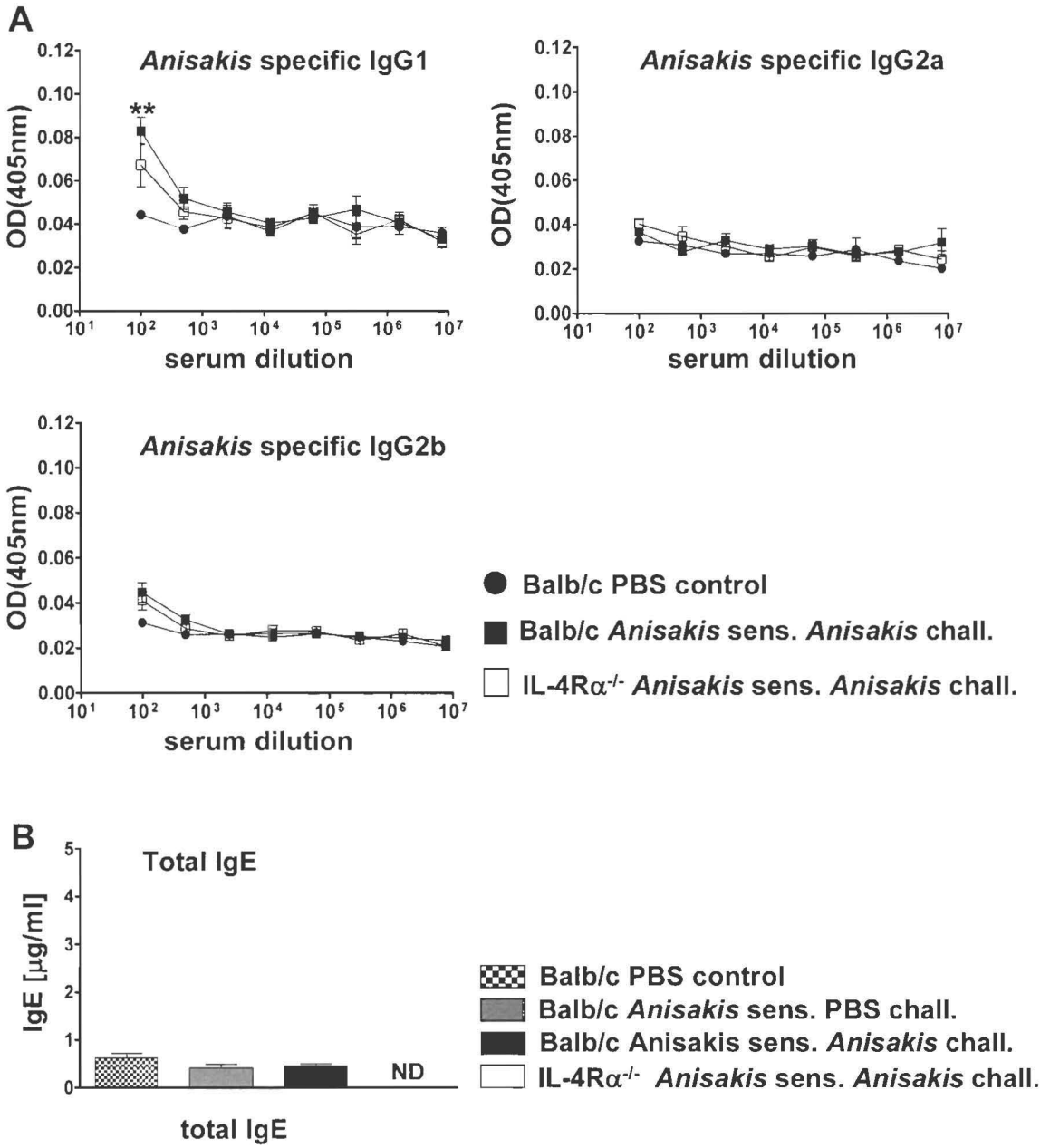

Figure 3. Nasal sensitisation with *Anisakis* antigens does not induce systemic IgE but weak IgG1 antibody response. Balb/c and IL-4Rα$^{-/-}$ mice were intranasally sensitised and challenged with *Anisakis* extract Serum concentrations of antigen specific IgG (A) total IgE (B) were determined by ELISA. Data represent the pool of two experiments +/-SD (**p<0.01 vs. PBS control group).

Anisakis specific IgE responses were compared by Western Blot analysis to identify *Anisakis* allergens (Fig. 4). IgE binding to *Anisakis* proteins was found only in the Balb/c strain but not in IL-4Rα$^{-/-}$ mice after live infection confirming the ELISA results where total IgE concentrations were only elevated in Balb/c mice. IgE antibodies against four allergens with the size of 35, 45, 70 and 73 kDa were found in Balb/c mice. No *Anisakis* specific antibodies were detected in PBS control mice.

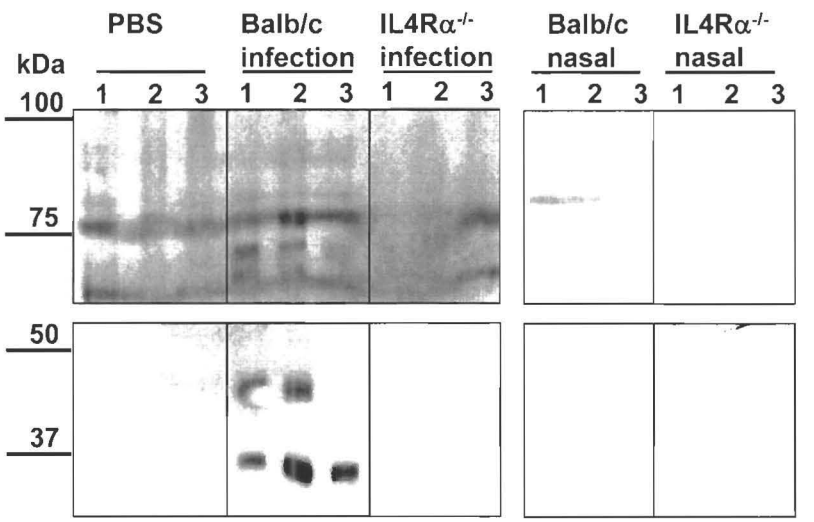

Figure 4. Infection with *Anisakis* larvae but not nasal sensitisation with *Anisakis* antigens induces IL-4Rα dependent IgE antibody response. Western blot analysis of serum from Balb/c mice or IL-4Rα$^{-/-}$ mice after *Anisakis* infection or nasal antigen sensitisation for *Anisakis* specific IgE antibody. (3 individual mice per group).

2.4.2 Allergen specific cytokine Response after intranasal allergen challenge

Development of allergic asthma is driven by Th2 cytokines especially IL-4, IL-5 and IL-13. Therefore lymphocytes from lung draining lymph nodes were analyzed for cytokine production after *in vitro* restimulation with *Anisakis* extract. In Balb/c mice infected with *Anisakis* larvae, intranasal antigen challenge induced an antigen specific Th2 cytokine response with increased levels of IL-4, IL-5 and IL-13 in lung draining lymph nodes (Fig. 5A). Cytokine

levels in infected and PBS challenged mice were not elevated when compared to the uninfected control group. IL-4Rα$^{-/-}$ mice responded with a predominant Th1 cytokine response to intranasal challenge after infection as reflected by highly increased IFN-γ only slightly elevated concentrations of the Th2 cytokines IL-4 and IL-5, and no detectable IL-13 (Fig. 5A).

A similar response was found after nasal sensitisation with *Anisakis* extract (Fig. 5B). Sensitisation to *Anisakis* extract alone did not induce a Th2 response in the lung draining lymph nodes. After intranasal challenge cells in lung draining lymph nodes of sensitized Balb/c mice produced Th2 cytokines including IL-4, IL-5 and IL-13, but not IFN-γ whereas IL-4Rα$^{-/-}$ mice produced IFN-γ but only low levels of IL-4 and IL-5, and no detectable IL-13.

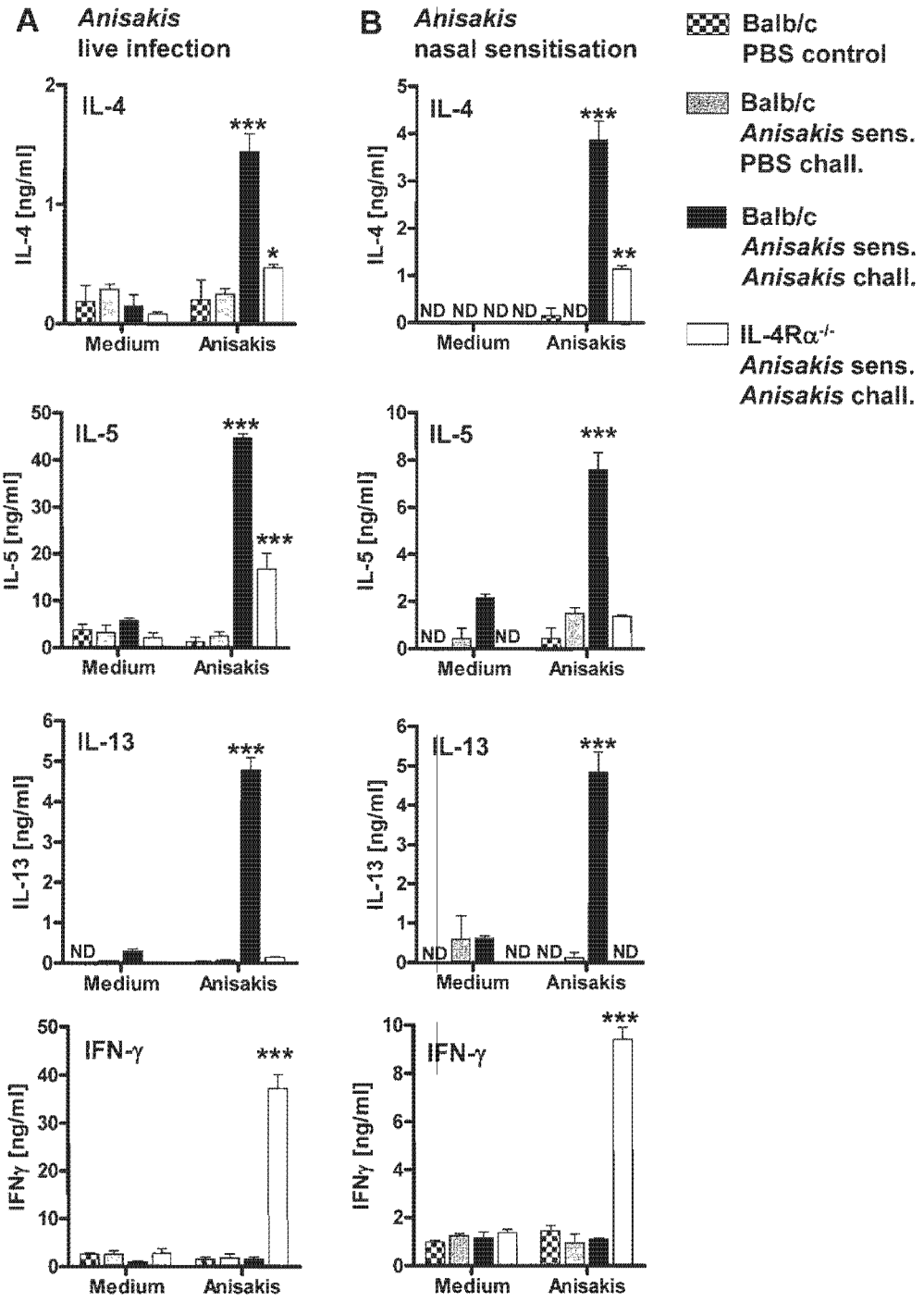

Figure 5. Balb/c mice develop an IL-4Rα dependent TH2 type cytokine response to Anisakis antigens. Lymphocytes were isolated from lung draining lymph nodes of mice challenged with Anisakis antigens after sensitisation by (A) live infection with *Anisakis* larvae or (B) nasal administration of *Anisakis* extract. Cytokine secretion of pooled lymphocytes *in vitro* stimulated with *Anisakis* extract or culture medium alone was measured by ELISA in triplicates. Data represent one of two experiments +/-SD (*p<0.05; **p<0.01; ***p<0.001 above medium control).

2.4.3 Airway hyperresponsiveness after intranasal allergen challenge

Airway hyperresponsiveness (AHR) to non-specific bronchoconstrictors is characteristic of allergic asthma. After *Anisakis* infection and challenge or intranasal sensitisation and challenge mice were exposed to increasing doses of aerosolized β-methacholine and airway hyperresponsiveness was determined by whole body plethysmography. Intranasal challenges induced airway hyperresponsiveness in Balb/c mice after infection with *Anisakis* larvae (Fig. 6A) and after nasal sensitisation with *Anisakis* extract (Fig. 6B) as reflected in increased Penh values after challenge with 20 and 40mg/ml β-methacholine. Interestingly AHR persisted in IL-4Rα$^{-/-}$ mice only in the model of *Anisakis* live infection (Fig. 6A). AHR after challenge with 20mg/ml β-methacholine was higher in IL-4Rα$^{-/-}$ mice compared to Balb/c mice and an equal response was found after 40mg/ml challenge. In the intranasal sensitisation/ challenge model development of AHR was IL-4Rα dependent and Penh values were higher for Balb/c mice compared to IL-4Rα mice (Fig. 6B). Sensitisation alone had no significant effect AHR as the response to β-methacholine was not significantly different between the PBS control group and Anisakis sensitized or infected Balb/c mice.

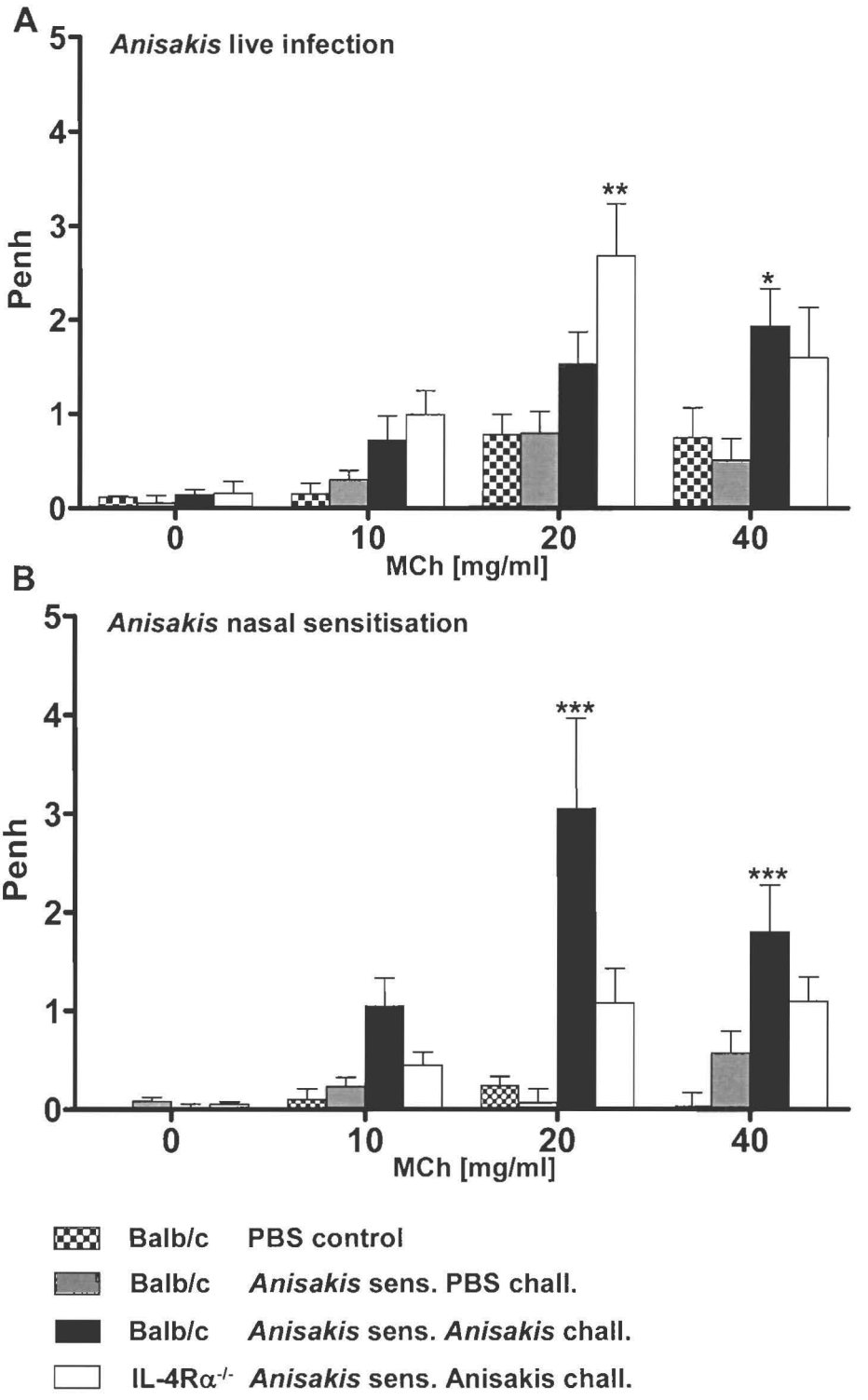

Figure 6. Airway hyperresponsiveness after intranasal challenge with *Anisakis* antigens is IL4Rα dependent after nasal sensitisation to *Anisakis* but not after live infection. Airway response to increasing concentrations of aerosolised methacholine (MCh) was measured by whole body plethysmography 1d after final allergen challenge in unrestrained mice sensitised by (A) live infection with *Anisakis* larvae or (B) nasal administration of *Anisakis* extract. Penh: Average of Penh values of the first 5min after MCh challenge. Data represent pool of two experiments +/-SD (*p<0.05; **p<0.01; ***p<0.001 vs. PBS control group).

2.4.4 Recruitment of inflammatory cells to the lungs of allergen challenged mice

In allergic asthma bronchi and alveoli are infiltrated by inflammatory cells with eosinophils being the characteristic cell type. Total numbers of inflammatory cells in the bronchoalveolar lavage of *Anisakis* challenged Balb/c and IL-4Rα$^{-/-}$ mice were significantly elevated above the PBS control groups in both models of *Anisakis* sensitisation (Fig. 7A). In the live infection model the total cell number in Balb/c mice was significantly higher than in IL-4Rα$^{-/-}$ mice (7.3 x10^5 vs. 3.7 x10^5). Differential cell counts revealed that the main cell type infiltrating the BAL in Balb/c mice was eosinophils whereas in IL-4Rα$^{-/-}$ mice it was neutrophils (Fig. 7B). The total number of eosinophils found in Balb/c mice was double the number of neutrophils found in IL4Rα$^{-/-}$ mice explaining the difference in total cell numbers between the two mouse groups. Number of lymphocytes increased slightly but not significantly and total number of macrophages did not change after allergen challenge. No differences in numbers of macrophages and lymphocytes in the BAL were detected between Balb/c and IL-4Rα$^{-/-}$ mice.

In the nasal sensitisation model no differences of total cell numbers was found between Balb/c and IL-4Rα$^{-/-}$ mice (Fig. 7A). As in the infection model the main cell type infiltrating the BAL in Balb/c mice was eosinophils whereas in IL-4Rα$^{-/-}$ mice it was neutrophils (Fig.7B). The eosinophil number in Balb/c was equal to the number of neutrophils in IL-4Rα$^{-/-}$ mice. Number of lymphocytes increased slightly but not significantly in both mouse strains and numbers of lymphocytes in the BAL were equal in both mouse strains. Total number of macrophages increased significantly in Balb/c but not in IL-4Rα$^{-/-}$ mice.

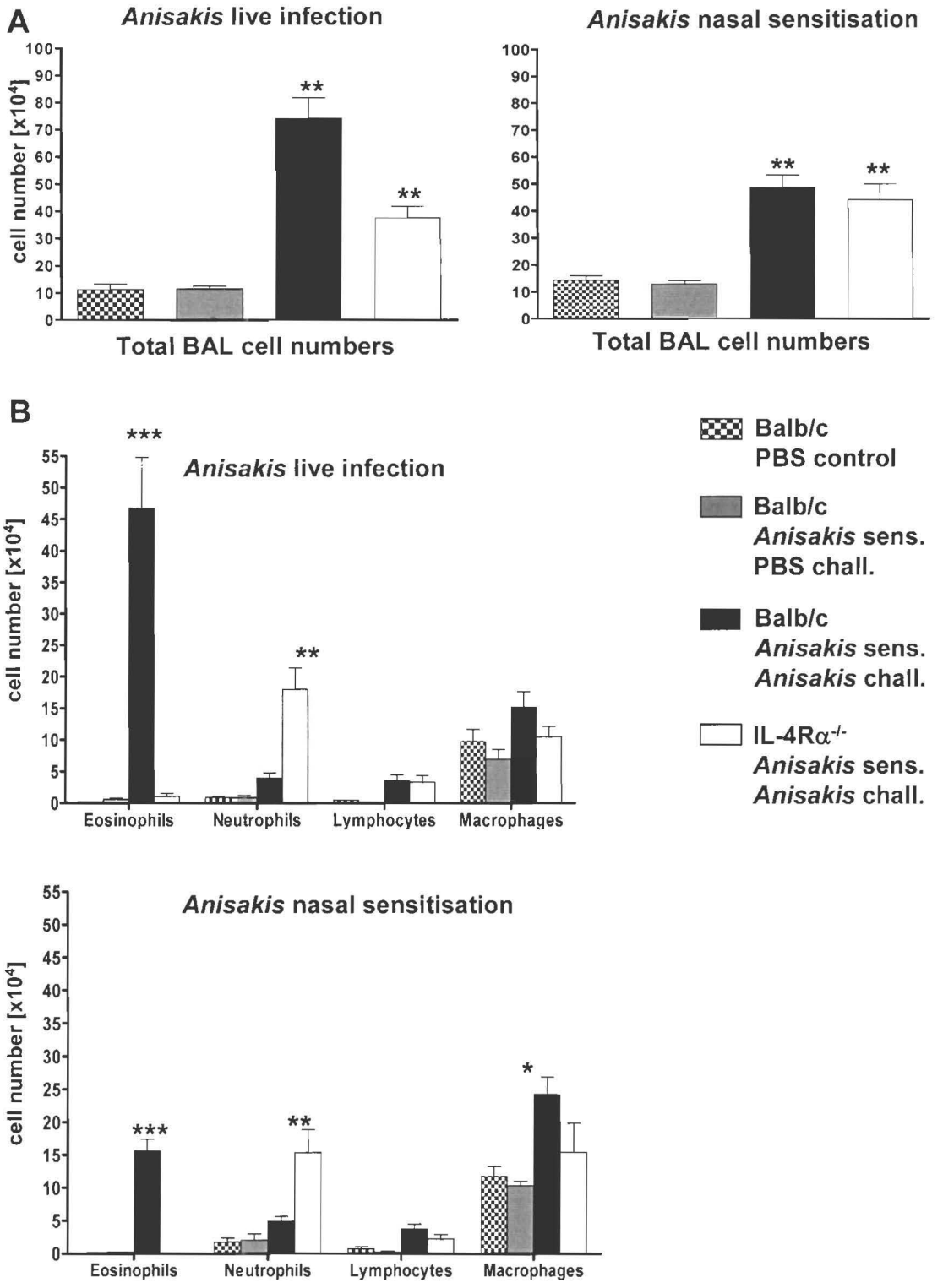

Figure 7. Lungs of sensitised mice are infiltrated by inflammatory cells after intranasal challenge with *Anisakis* antigens. Total cell numbers (A) and cellular composition (B) in BAL fluid was determined after challenge of sensitised mice with *Anisakis* extract. Data represent pool of two experiments for each sensitisation protocol+/-SD (*p<0.05; **p<0.01***p<0.001 vs. PBS control group).

2.4.5 Lung pathology after airway challenge with *Anisakis* extract

During allergic airway reactions, inflammatory cells infiltrate the lung tissue around blood vessels and airways. PBS sensitisation and challenge (Fig. 8A, E) and live infection and nasal sensitisation alone (Fig. 8B, F) failed to induce infiltration of the lungs by inflammatory cells. After intranasal challenge with *Anisakis* extract Balb/c mice (Fig. 8C, G) and IL-4R$\alpha^{-/-}$ mice (Fig. 8D, H) showed perivascular and peribronchial inflammation in both models of *Anisakis* sensitisation.

Hyperplasia of mucus producing goblet cells in the lungs is a characteristic of allergic asthma. PBS sensitised and challenged mice showed no goblet cell hyperplasia (Fig. 8A, E). Histological examination of PAS stained lungs of Balb/c mice challenged with *Anisakis* protein extract after infection or intranasal challenge showed similar severity of goblet cell hyperplasia (Fig. 8C, G) whereas *Anisakis* infection or antigen sensitisation alone did not induce increased mucus production (Fig. 8B, F). Mucus production was dependent on IL-4/ IL-13 signals as goblet cell hyperplasia was absent in IL-4R$\alpha^{-/-}$ mice (Fig. 8D, H).

H&E staining of lung sections showed high numbers of eosinophils present in the inflammatory cell infiltrate in Balb/c after *Anisakis* infection and nasal sensitisation (Fig. 9C, G). In IL4R$\alpha^{-/-}$ mice airway inflammation was present but without the eosinophilia seen in Balb/c mice (Fig. 9D, H). Airway eosinophilia was not found in PBS control mice (Fig. 9A, E), and after nasal sensitisation or Anisakis infection followed by PBS challenge (9B, F).

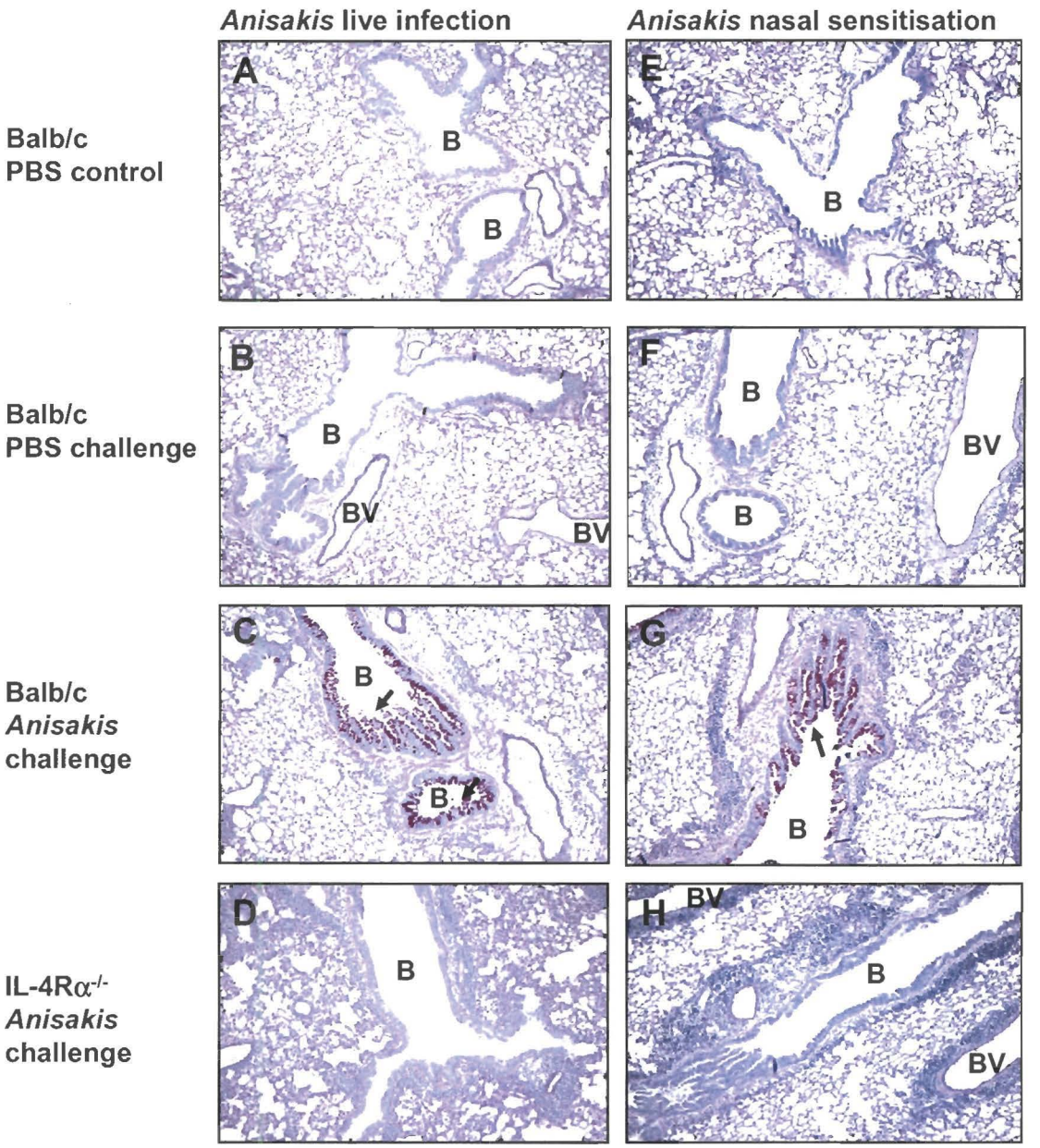

Figure 8. Goblet cell hyperplasia is induced after intranasal challenge with *Anisakis* antigens in Balb/c but not IL-4Rα$^{-/-}$ mice after live infection with *Anisakis* larvae and nasal sensitisation with *Anisakis* extract. Histology sections of lungs were stained with periodic acid Schiff (PAS) to visualise mucus producing goblet cells in the airway epithelium (arrows). 100x magnification. B: bronchiole; BV: blood vessel; Data are representative of two independent experiments.

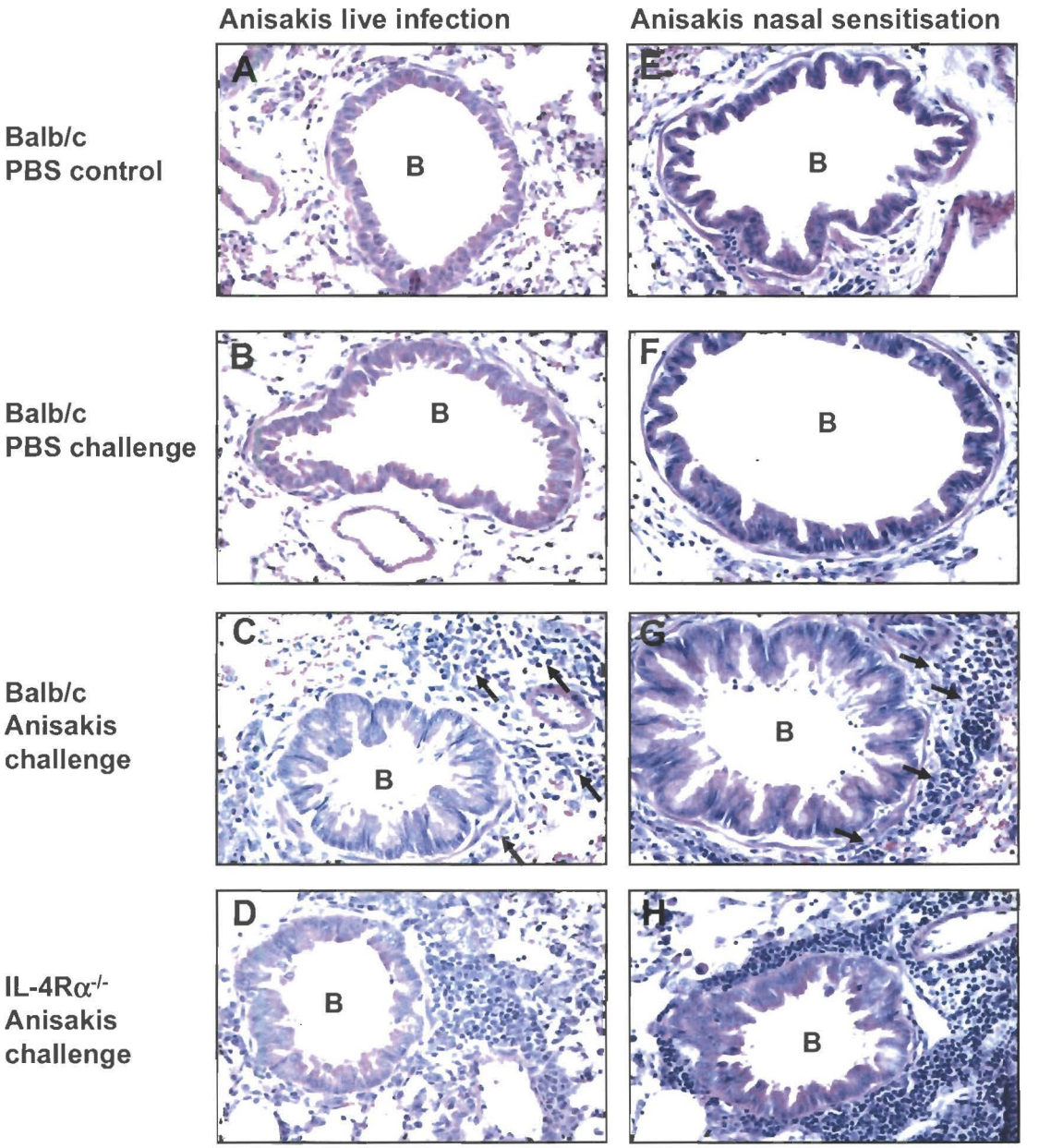

Figure 9. Eosinophilic airway inflammation after intranasal challenge with Anisakis antigens is dependent on IL-4Rα expression. Histology sections of lungs were stained with hematoxylin and eosin (H&E) to visualise infiltrating eosinophils (arrows). B: bronchiole; 400x magnification, Data are representative of two independent experiments.

2.5 Discussion

In the human context of *Anisakis* allergies, live infection seems to be the most common way of sensitization[11, 53] whereas sensitization by inhaling *Anisakis* proteins might be predominantly important for sensitisation in the occupational environment.[30, 35, 37] The aim of this study was to investigate whether different routes of sensitization to *Anisakis pegreffii* can influence the outcome of experimental allergic asthma and underlying immune responses. Two new mouse models of allergic asthma were established for this study in order to address this question.

A mouse model of infection with live *Anisakis pegreffii* larvae was compared to a model of sensitization to *Anisakis* extract via the nasal route. After sensitization mice were challenged intranasally with *Anisakis* proteins to induce allergic responses in the airways. The identical method of allergen challenge enabled the investigation of differences in the pathology of the disease that were due to different routes of sensitization. Furthermore, the contribution of signalling through IL-4Rα, an important receptor in the regulation of allergic asthma in mouse models and the human disease,[54] was taken into account by comparing wild type Balb/c mice with IL-4Rα$^{-/-}$ mice. This study with its focus on allergic airway responses in *Anisakis* allergies is in context of previous work in our research group where immune responses after *Anisakis* infection and gastric allergen exposure were investigated. In the previous study infection with *Anisakis* larvae induced a Th2 type immune response and IL-4Rα dependent food-allergic symptoms.[30]

Comparison of signs of allergic asthma revealed that allergic airway disease was induced after live infection and nasal sensitization. Wild type Balb/c mice developed airway hyperresponsiveness, eosinophilic airway inflammation and hyperplasia of mucus producing goblet cells after challenge with *Anisakis* proteins. Onset of eosinophilic airway inflammation and goblet cell hyperplasia was IL-4Rα dependent as mice deficient for the receptor were protected against these symptoms. This is in agreement with observations from mouse

models which showed critical involvement of this receptor and its ligand IL-13 for allergen induced mucus production.[55, 56] Development of airway hyperresponsiveness in IL-4Rα$^{-/-}$ mice was dependent on the route of sensitisation as it was only observed after infection with live *Anisakis* larvae but not after nasal sensitisation. AHR in these mice came as a surprise as studies in mouse models of allergic asthma have shown a critical role for IL-4Rα and IL-13 in the development of airway hyperresponsiveness.[46, 47] However other studies have suggested IL-4Rα independent mechanisms of AHR in murine models of ovalbumin induced allergic asthma.[57-59] The nature of this mechanism has not been defined yet but it was suggested that IL-5 and IL-13 are essential for IL-4Rα independent AHR.[58] Whereas IL-5 was produced after live infection with *Anisakis*, no IL-13 production was found in lung draining lymph nodes of IL-4Rα$^{-/-}$ mice and the mechanism behind the development of AHR remains elusive. Differences between live infection and sensitisation with *Anisakis* extract include the exposure of the mice to the parasite over a long period (up to 14 days) and the *in vivo* secretion of proteins by the worm which are absent in the extract. These differences might be responsible for AHR development in IL-4Rα$^{-/-}$ mice after live infection but not after nasal sensitisation.

Airway inflammation in Balb/c mice showed typical signs of an allergic response such as eosinophil infiltrations around the airways and elevated eosinophil numbers in the bronchoalveolar lavage fluid. Comparing numbers of eosinophils in the BAL between the two sensitization groups revealed that live infection induced stronger eosinophilia than nasal sensitisation. Infection with *Anisakis* is accompanied by blood and tissue eosinophilia[26-28] which can explain the presence of high numbers of eosinophils in the allergic lung. *Anisakis* extract has been described to be chemotactic for eosinophils[29] but might be not as efficient as in recruiting eosinopils as the live worm. Another reason might be lower production of IL-5, important for eosinophil differentiation and migration, in the local lymph nodes after nasal sensitisation when compared to live infection.

After live infection, wild type and IL-4Rα$^{-/-}$ mice developed a strong IgG response against *Anisakis* antigens. This response was moderate three weeks after the first infection but a strong memory response was induced by reinfection and antibody levels increased substantially. In wild type mice this was the case for the type 2 antibodies IgG1 and IgE. IL-4Rα$^{-/-}$ mice on the other hand showed a mixed type 1/ type 2 antibody responses and the predominant antibody isotypes were IgG 2a and IgG2b, typically associated with Th1/ type 1 immune responses, but also the type 2 antibody IgG1. IgE could not be detected in these mice. A different situation was found after nasal sensitization where *Anisakis* specific antibodies were barley detectable despite the development of asthmatic symptoms. Balb/c mice produced low amounts of specific IgG1 with concentrations about 1000x lower compared to live infection and no IgE antibodies were found. Allergic airway symptoms in the absence of IgE have been described in murine models for aerosolised allergens [60, 61] and in human patients with asthma.[62, 63] Patients with non-IgE mediated asthma can develop respiratory symptoms in the absence of allergen specific IgE antibodies but show elevated IL-4, IL-5 and IL-13 levels.[64-66]

In contrast to the antibody response, cytokine production was not dependent on the route of allergen sensitization. Wild type mice developed an antigen specific Th2-type cytokine response in lung draining lymph nodes after nasal sensitisation and live infection. Cytokine responses in Balb/c mice were characterized by the production of IL-4, IL-5 and IL-13 but not IFN-γ after allergen challenge. The data from the nasal sensitization model suggests that differentiation of T cells into allergen specific Th2 cells was sufficient for the development of allergic airway symptoms in the absence of specific antibodies. This might be important for the diagnosis of *Anisakis* allergies which often relies on the identification of specific IgE[67-69]. IL-4Rα$^{-/-}$ mice showed a Th1 polarized response reflected by IFN-γ secretion, but did not develop allergic asthma after nasal sensitisation. The cytokine responses in both sensitization models were induced by the intranasal allergen challenge as PBS challenge of sensitized mice did not result in elevated cytokine levels.

As described previously,[70, 71] infection of mice with *Anisakis* larvae was temporary and no live or dead larvae were found three weeks after the second infection when mice were killed and analysed. Therefore all results described here for the live infection model as well as the nasal sensitization model represent a situation where no live parasite was present at the time of the onset of allergic airway symptoms. It is still debated whether the parasite has to be alive to induce allergic reactions [3] but in murine models at least, presence of *Anisakis* proteins appears be sufficient for the development of allergic reactions. This is in agreement with other studies of mouse models [30] and observations in humans.[17, 19]

This is the first study that specifically investigates the immune response in *Anisakis pegreffii* induced allergic asthma by using animal models. Development of airway symptoms after *Anisakis* live infection followed by gastric allergen challenge has been described previously and the present study shows that allergic airway inflammation can also be induced by exposure of the respiratory system to *Anisakis* allergens. Two unexpected findings were of particular interest and might reflect typical features of *Anisakis* induced allergic airway disease. Depending on the route of sensitisation, mice developed signs of the disease in the absence of either allergen specific antibodies or an allergen specific TH2 type immune response. Wild type mice developed a Th2 type immune response and typical allergic symptoms after infection with *Anisakis* larvae. In IL-4R$\alpha^{-/-}$ mice, AHR could also be induced in an IL-4Rα and Th2 independent manner. Furthermore, it was shown that non-IgE mediated allergic asthma was induced after sensitization to *Anisakis* via the nasal route.

The results presented here show new aspects of the immunology of *Anisakis* related allergies and demonstrate the importance of the route of sensitisation to *Anisakis pegreffii* for the development of allergic airway inflammation and its underlying immune response. Further studies are necessary to understand specific aspects of the disease in order to develop adequate diagnosis and treatment strategies for *Anisakis* allergies.

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Chapter 3

Smooth muscle IL-4Rα is not required for experimental allergic asthma

3 Smooth muscle IL-4Rα is not required for experimental allergic asthma

3.1 Summary

Background

Airflow in the lungs of patients with allergic asthma is impaired by excessive mucus production and airway smooth muscle contractions. Elevated levels of the cytokines IL-4 and IL-13 are associated with this pathology. *In vitro* studies have suggested that IL-4Rα signalling on smooth muscle cells is critical for airway inflammation and airway hyperresponsiveness. By using transgenic SMC-MHC^{cre}IL-4Rα$^{-/lox}$ mice deficient for IL-4Rα in smooth muscle cells, *in vivo* effects of impaired IL-4Rα signalling in smooth muscle cells on the outcome of asthmatic disease were investigated for the first time.

Methods

Allergic asthma was introduced in mice by repeated sensitisation with ovalbumin/aluminium hydroxide on days 0, 7 and 14 followed by intranasal allergen challenge on days 21-23. Mice were investigated for the presence of airway hyperresponsiveness, airway inflammation, allergen specific antibody production, Th2 type cytokine responses and lung pathology.

Results

Airway inflammation, airway hyperresponsiveness, mucus production, Th2 cytokine production and specific antibody responses were unaffected in SMC-MHC^{cre}IL-4Rα$^{-/lox}$ mice when compared to control animals. Unexpectedly, the impairment of IL-4Rα on smooth muscle cells had no effect on major aetiological markers of allergic asthma.

Conclusion

These findings suggest that IL-4Rα responsiveness in airway smooth muscle cells during the early phase of allergic asthma is not, as suggested, critical for the outcome of the disease.

3.2 Introduction

Allergic asthma is characterised by airway obstruction, inflammation, and airway hyperresponsiveness (AHR). Patients with allergic asthma experience impaired respiratory air flow which is caused by a combination of increased airway mucus secretion and airway smooth muscle cell (ASMC) contraction in response to allergens.

Immunologically it is established that T helper 2 (Th2) type cytokines, particularly IL-4 and IL-13 play an important role in asthma pathogenesis. Indeed, overexpression of these cytokines results in the development of asthmatic symptoms in murine models.[1-4] Though IL-4 plays an important role in establishing Th2 responses,[5, 6] IL-13 is the cytokine critical for driving actual asthmatic pathology. In the absence of IL-13, mucus production, AHR and airway inflammation are inhibited. Conversely, administration of recombinant IL-13 will induce these pathological features in non allergic mice.[7, 8] Such IL-13 driven effects occur irrespective of IL-4, nevertheless induction of a strong Th2 response and immunoglobulin class switching to IgE in mice requires IL-4. These IL-4 and IL-13 mediated effects require signalling through heterodimeric receptors containing IL-4 receptor alpha (IL-4Rα). Mice deficient for IL-4Rα show protection from experimentally induced allergic asthma [9] and IL-4Rα antagonists abrogate the development of allergic airway symptoms.[10]

It can be appreciated from this that an understanding of the role of IL-4Rα in specific cell types would enhance the understanding and treatment of asthma. Key cells involved in the onset and pathogenesis of asthma include T cells, B cells, epithelial and smooth muscle cells. To date the cell specific role of IL-4Rα in allergy has only been described *in vivo* for epithelial cells. Here disrupted epithelial IL-4Rα signalling results in protection from AHR and airway mucus production.[11]

In addition to immune and epithelial cells it is apparent that ASMC may also play an important role in the onset of asthma. Several *in vitro* studies have shown that IL-4 and IL-13 induce hypercontractility in smooth muscle cells in a STAT6 dependent manner.[12-14] This IL-4 and IL-13 dependent

hypercontractility response of ASMC to carbachol was attenuated in STAT6$^{-/-}$ mice. Use of anti-IL-4Rα antibodies also inhibited the contractile responsiveness of IgE sensitised ASMC to acetylcholine while pre-treatment with recombinant IL-13 enhanced contractile ability.[15] In addition to effects on ASMC hypercontractility, human airway smooth muscle cells have been shown to release allergy associated chemokines, such as eotaxin [16-22] in response to IL-4 and/ or IL-13 *in vitro*. This IL-4/IL-13 driven eotaxin expression in ASMC can be inhibited by anti-IL4Rα antibodies [17] and antisense oligonucleotides to STAT6.[23]

Owing to this substantial body of *in vitro* data, it was suggested that ASMC responsiveness to IL-4 and IL-13 contributes significantly to asthmatic pathology. To investigate the *in vivo* role of IL-4Rα signalling in smooth muscle cells we used a mouse model with smooth muscle cell specific disruption of the *Il4ra* gene (SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice). Recently characterised by our laboratory, SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice showed delayed mucus production, Th2 cytokine production and worm expulsion in a model of nematode infection.[14] This was the first *in vivo* demonstration of the influence of IL-4Rα expression in smooth muscle cells on Th2 type immune responses of the host.

The current study investigated for the first time the *in vivo* effect of IL-4Rα signalling in smooth muscle cells in a model of acute allergic asthma. The findings of several *in vitro* studies have suggested a role for IL-4/IL-13 responsiveness in smooth muscle cells on the outcome of allergic airway disease, but surprisingly our results demonstrated that smooth muscle cell specific disruption of the IL-4Rα did not influence the acute phase of the allergic airway response caused by ovalbumin (OVA).

3.3 Materials and Methods

3.3.1 Mice

Generation and characterization of the SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice was described previously.[14] SM-MHC^{cre} mice on a BALB/c background [24] were crossed with IL-4R$\alpha^{-/-}$ mice.[25] In order to generate SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice they were then crossed with IL-4R$\alpha^{-/lox}$ mice [26] as the hemizygous IL-4R$\alpha^{-/lox}$ genotype increases the probability of Cre-mediated *il4ra* gene disruption. All mice were genotyped by PCR before the experiments to confirm smooth muscle cell-specific disruption of the *il4ra* gene. IL-4R$\alpha^{lox/lox}$ mice produce a functional IL-4Rα [26] and hemizygous IL-4R$\alpha^{-/lox}$ littermates were used as control groups. IL-4R$\alpha^{-/-}$ mice were used as global knock out controls for the IL-4Rα. Mice were housed in the animal unit of the University of Cape Town (UCT) under specific pathogen free conditions using individually ventilated cages. All work was approved by the UCT animal ethics committee.

3.3.2 Isolation of smooth muscle cells from lung tissue

Lungs were removed from mice, flushed with PBS, cut into small pieces and incubated for 3h at 37°C in DMEM containing 1mg/ml Collagenase type II (SIGMA-ALDRICH, Germany). Digested tissue was put through a 70μm cell strainer and erythrocytes were lysed in a 0.15M NH_4Cl buffer. For intracellular α-actin staining cells were incubated in Fixation/Permeabilization buffer (eBioscience, USA), blocked with 2% normal rat serum and 2% Fc block (2.4G2) and stained with anti-smooth muscle α-actin FITC (Abcam, UK) or isotype control IgG2a FITC (BD, USA) antibodies. FITC positive cells were sorted and analysed for purity using a FACSVantage cell sorter (BD, USA).

3.3.3 Analysis of IL-4Rα deletion efficiency by RT-PCR

Genomic DNA was isolated from trachea and spleen tissue, $CD4^+$ lymphocytes and α-actin sorted lung cells of SM-MHC^{cre}IL-4$R\alpha^{-/lox}$, IL-4$R\alpha^{-/lox}$ and IL-4$R\alpha^{-/-}$ mice. The copy numbers of IL-4Rα exon 5 and IL4-Rα exon 8 were determined using a Roche Lightcycler (Roche, Germany) and amplification was monitored using SYBR GreenI. Cloned IL-4Rα exon 5 and exon 8 DNA was used to prepare a standard curve. Primers: exon 5 forward 5'AACCTGGGAAGTTGTG 3', exon 5 reverse 5' CACAGTTCCATCTGGTAT 3', exon 8 forward 5'GTACAGCGCACATTGTTTTT 3' and exon 8 reverse 5' CTCGGCGCACTGACCCATCT 3'.

3.3.4 Nippostrongulus Brasiliensis infection of mice

Mice were infected with 750 *N.brasiliensis* L3 larvae as described previously and lung tissue was analysed at days 7 and 10 post infection. [14]

3.3.5 Sensitisation and Challenge of mice

Sensitisation and challenge of mice was performed as described previously.[11] SM-MHC^{cre}IL-4$R\alpha^{-/lox}$, IL-4$R\alpha^{-/lox}$ and IL-4$R\alpha^{-/-}$ mice were sensitised with 50 μg Ovalbumin (Sigma-Aldrich, grade V) in 200μl PBS/ 1.3% Alum (Sigma-Aldrich) on day 0, day 7 and day 14. On day 21, 22 and day 23, mice were anaesthetised with Ketamine (Centaur Labs, South Africa)/ Xylazine (Bayer, South Africa) and challenged with 1mg OVA in 50μl PBS by nasal administration. Control groups were treated identically except OVA was missing in the solutions. Mice were killed and studied on day 24.

3.3.6 Measurement of Airway Hyperreactivity

Airway reactivity measurements were performed as described previously.[11] Anaesthetised and paralysed mice were mechanically ventilated and lung resistance was measured at baseline and following increasing intravenous doses of acetylcholine.

3.3.7 Analysis of airway inflammation

Lungs from sacrificed mice were flushed once with 1ml PBS/1% FCS to obtain bronchoalveolar lavage (BAL) fluid and total cell number was determined using a haemocytometer. For histological analysis, tissue samples were fixed in 4% phosphate-buffered formalin, cut in 5-7µm paraffin sections and stained with Periodic acid-Schiff reagent (PAS) or haematoxylin and eosin (H&E). Lung tissue was homogenized in Tris/ NP-40 buffer and then centrifuged for 20 minutes at 20000rcf for mouse mast cell protease-1 (mMCP-1) ELISA and eosinophil Peroxidase (EPO) assay. A colorimetric assay for EPO was used to quantify eosinophils in the lung tissue.[27] 50 µl of the substrate solution consisting of 16mM o-phenylenediamine (Sigma-Aldrich) in 100mM Tris-HCl buffer pH 8.0 containing 0.1% Triton and 0.01% hydrogen peroxide was added to 50 µl tissue sample (protein concentration 1mg/ml) and peroxidase standard (Sigma-Aldrich) in 96 well plates. The reaction was stopped after 5 minutes with 50µl 2M H_2SO_4 and absorption was measured at 490nm. Mouse mast cell protease concentrations were determined by ELISA (Moredum Scientific, UK) according to the manufacturer's protocol. Serum eotaxin levels were determined by ELISA according to the manufacturer's protocol (R&D Systems, USA).

3.3.8 Measurement of serum antibodies

Blood samples were collected in plasma separator tubes (BD, USA) by tail vein bleeding and centrifuged for 15 minutes at 6000rcf. OVA-specific antibody isotypes were measured in blood serum by endpoint titration ELISA. Plates were coated with 5µg/ml (for IgG) or 1mg/ml (for IgE) OVA. AP-conjugated anti-mouse isotype specific antibodies (Southern Biotechnology, USA) and 4-nitrophenyl phosphate (Fluka, CH) were used for detection. Absorbance was measured at 405nm with 492nm as a reference wavelength.

3.3.9 Analysis of in vitro cytokine production

For analysis of *in vitro* cytokine production splenocytes and lymphocytes were isolated from pooled spleens or mediastinal lymph nodes of OVA treated and control mice. Cells were incubated for 120h at 1x 10^6 cells/ml in 200µl IMDM (Gibco, UK)/10% FCS (Delta, South Africa) in 96well plates. Cells were stimulated with anti-CD3 antibodies (clone 145-2C11; 10µg/ml) and supernatants were collected after 120 hours. Concentrations of IL-4, IL-5, IL-9, IFNγ (BD Biosciences, USA) and IL-13 (R&D Systems, USA) were measured using sandwich ELISA assays according to the manufacturer's protocol.

3.3.10 Statistics

Values are given as mean ± SD and significant differences determined using Student's t-test (Prism software). P values ≤ 0.05 were considered significant.

3.4 Results

3.4.1 Disruption of the il4ra gene in the airways of SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice

Smooth muscle cell specific disruption of the *il4ra* gene is achieved by cell specific expression of Cre recombinase. Here Cre expression excises the *loxP* flanked exons 7-9 from the *il4ra* gene in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice (Fig.1A). Disruption of the *il4ra* gene was analysed at the genomic level by comparing the ratio of the exon 8 and exon 5 alleles in SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ control mice by quantitative real time-PCR.

Exon 8 was significantly reduced in tracheal genomic DNA in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice when compared to IL-4Rα$^{-/lox}$ mice ($p= 0.0407$). In the spleen no differences were found in exon 8/exon 5 ratio between SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice (Fig.1B). To confirm specificity of gene disruption, α-actin enriched cell samples were obtained from lungs and trachea by cell sorting. The percentage of α-actin positive cells after purification was 69.94% for the SM-MHC^{cre}IL-4Rα$^{-/lox}$ and 78.66% for the IL-4Rα$^{-/lox}$ mice. Analysis of smooth muscle cell enriched samples revealed a significant reduction of the ratio of exon 8 to exon 5 in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice when compared to IL-4Rα$^{-/lox}$ mice ($p= 0.0036$). In purified CD4^{+} T cells the exon 8 to exon 5 ratio was equivalent in both mouse groups (Fig.1C).

The effect of *il4ra* smooth muscle cell specific gene disruption was demonstrated by infection studies with the nematode *Nippostrongylus brasiliensis*. Such infections induce a Th2 dependent pulmonary pathology characterised by heightened airway mucus production.[28, 29] In this study SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice were found to have reduced mucus production at days 7 and 10 post infection when compared to IL-4Rα$^{-/lox}$ mice. IL-4Rα$^{-/-}$ mice showed no signs of airway mucus production in response to the nematode infection (Fig.1D).*

Taken together these results demonstrate a functional smooth muscle cell specific disruption of the *il4ra* gene in the airways of SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice and agree with previously published results on the characterisation of this mouse.[14]

*Data for *Nippostrongylus brasiliensis* infection provided by William G. Horsnell

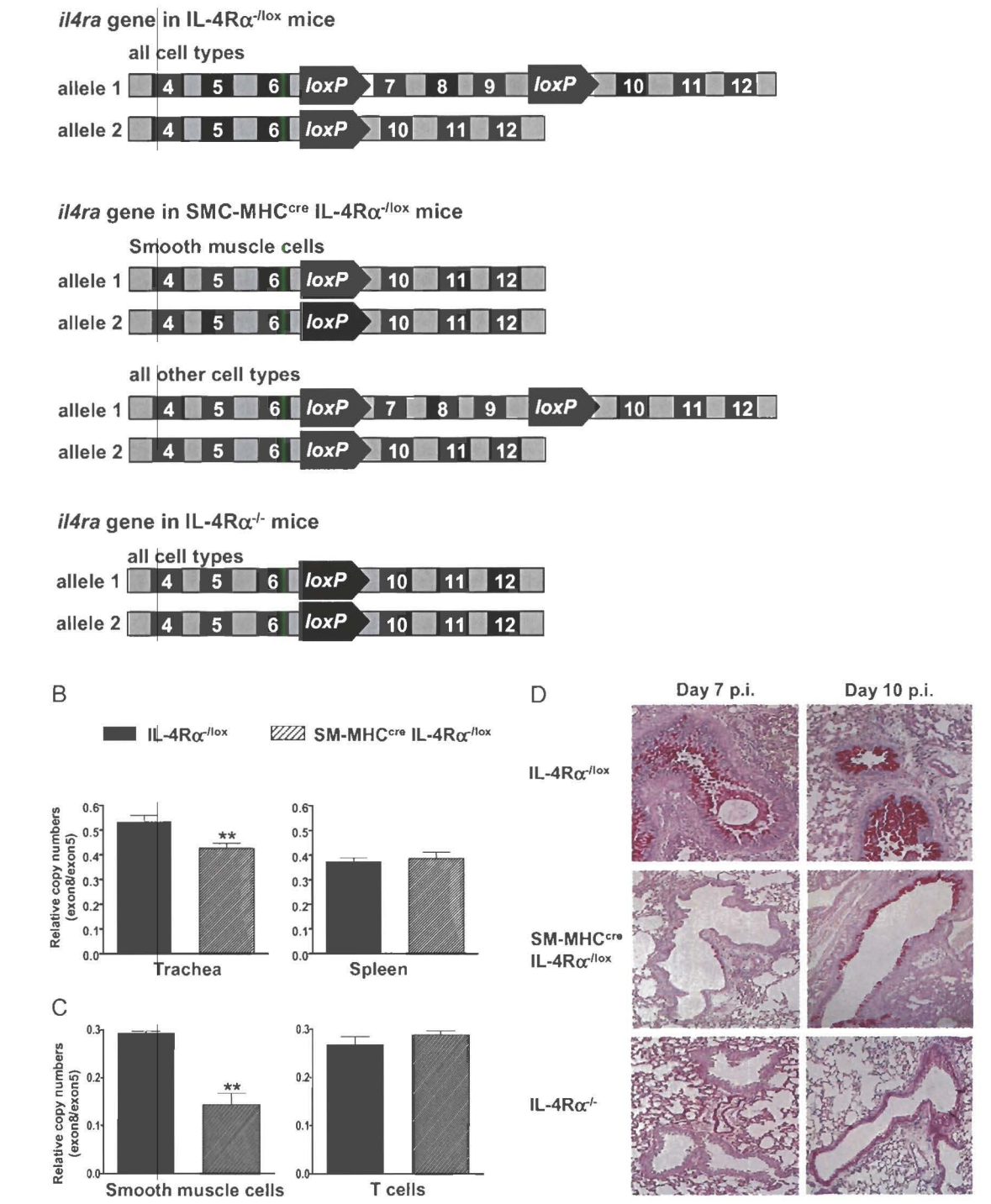

Figure 1. IL-4Rα deletion in smooth muscle cells of SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice. Black boxes represent exons of the *il4ra* gene locus in IL-4Rα$^{-/lox}$, SM-MHC^{cre} IL-4Rα$^{-/lox}$ and IL4Rα$^{-/-}$ mice (A). The ratio of IL-4Rα exon 8 to exon 5 alleles was determined by real-time PCR in genomic DNA samples from trachea and spleen tissue from IL-4Rα$^{-/lox}$ and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice (B). Smooth muscle cells from lung tissue were sorted after intracellular α-actin staining versus Isotype control. The ratio of IL-4Rα exon 8 to exon 5 alleles was determined by real-time PCR in genomic DNA samples from sorted smooth muscle cells and CD4^{+} T cells from IL-4Rα$^{-/lox}$ and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice (C). Mucus producing goblet cells were stained with PAS in the airways of IL-4Rα$^{-/lox}$, SM-MHC^{cre} IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice on day 7 and day10 post infection (p.i.) with *N. brasiliensis* (D); (**, p< 0.005)

3.4.2 Allergen-induced airway hyperreactivity is not affected in SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice

Airway reactivity was measured in sedated and paralyzed, mechanically ventilated SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice after OVA or saline challenge. Airway resistance was increased in allergen-challenged IL-4R$\alpha^{-/lox}$ and SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice, indicating the development of airway hyperreactivity. These increases in airway resistance were not observed in saline challenged mice. Similar increases in airway reactivity were seen inSM-MHC^{cre}IL-4R$\alpha^{-/lox}$ and IL-4R$\alpha^{-/lox}$ mice (Fig.2).**

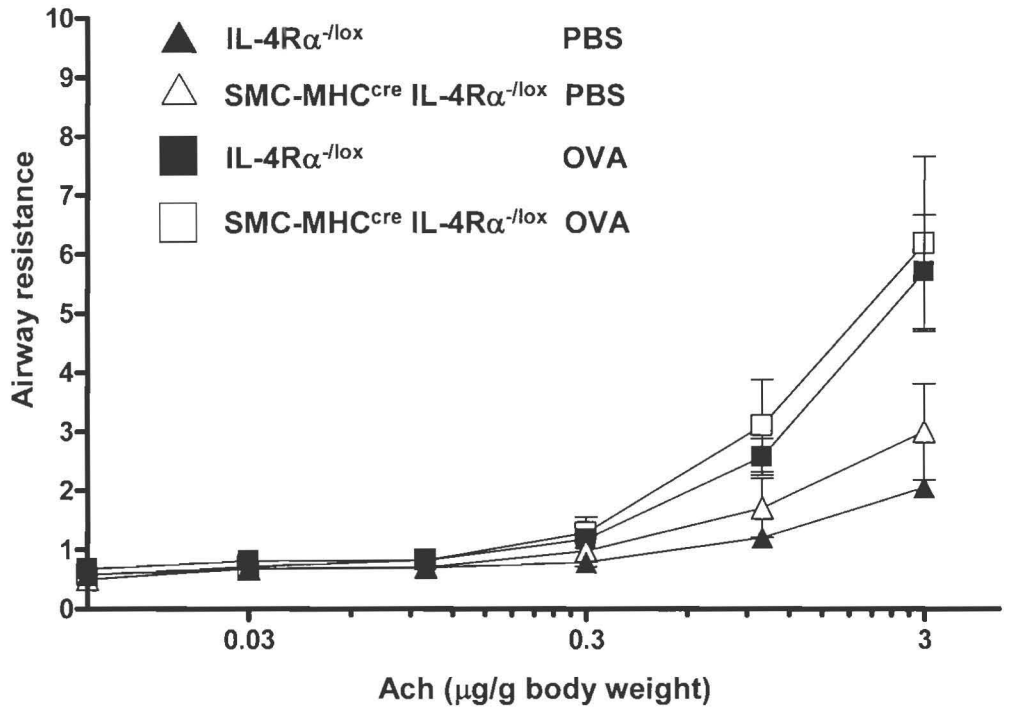

Figure 2. Airway resistance is not affected by loss of the IL-4Rα on smooth muscle cells. Airway response to increase concentrations of Acetylcholine was measured in IL-4R$\alpha^{-/lox}$ and SM-MHC^{cre} IL-4R$\alpha^{-/lox}$ mice after OVA or PBS sensitization and challenge.

**Data provided by Douglas A. Kupermann , Xiaozhu Huang and David J. Erle

3.4.3 Airway infiltration with inflammatory cells and mucus production are not affected in SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice

During allergic airway reactions, inflammatory cells infiltrate the lung tissue around blood vessels and airways. The alum treated control group failed to demonstrate any infiltration of the lungs by inflammatory cells (Fig. 3A). Both, IL-4R$\alpha^{-/lox}$ mice and SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice showed severe perivascular and peribronchial inflammation with eosinophil infiltration (Fig 3B,C), whereas IL-4R$\alpha^{-/-}$ mice showed mild signs of inflammation (Fig.3D). Hyperplasia of mucus-producing goblet cells in the lungs is a characteristic of allergic asthma. Histological examination of PAS stained lungs of OVA sensitized and challenged IL-4R$\alpha^{-/lox}$ mice and SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice showed similar severity of goblet cell hyperplasia (Fig. 3F,G), whereas the alum treated control group and IL-4R$\alpha^{-/-}$ mice showed no signs of increased mucus production (Fig. 3E, H).

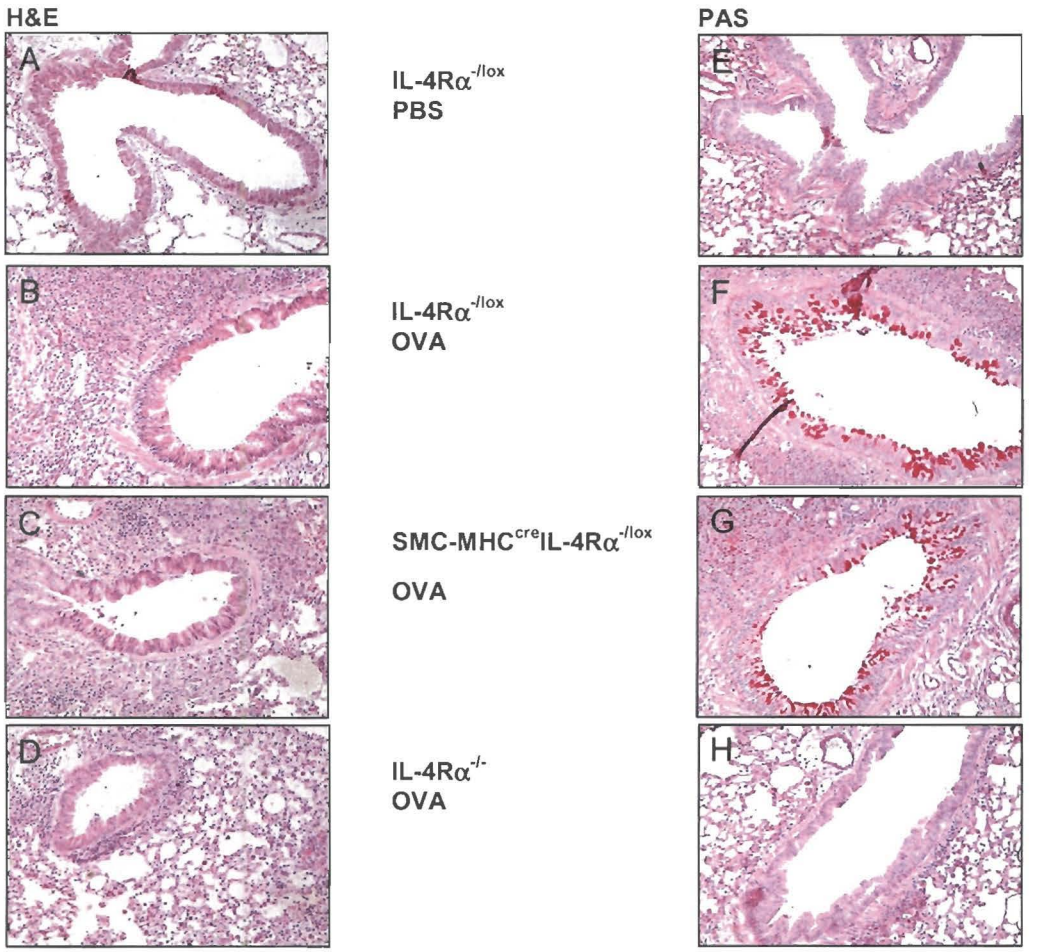

Figure 3. Infiltration of the lung tissue with inflammatory cells and goblet cell hyperplasia after sensitisation and challenge with OVA is not affected by loss of the IL-4Rα on smooth muscle cells. Lung sections from IL-4Rα$^{-/lox}$ mice sensitized and challenged with PBS (A) and OVA sensitised and challenged IL-4Rα$^{-/lox}$ (B), SM-MHC^{cre}IL-4Rα$^{-/lox}$ (C) and IL-4Rα$^{-/-}$ mice (D) were stained with H&E. Mucus producing goblet cells were stained with PAS in the airways of PBS sensitized and challenged IL-4Rα$^{-/lox}$ mice (E) and OVA sensitized and challenged IL-4Rα$^{-/lox}$ (F), SM-MHC^{cre} IL-4Rα$^{-/lox}$ (G) and IL-4Rα$^{-/-}$ mice (H). Pictures were taken with a magnification of x200 and representative examples of two experiments are shown here.

3.4.4 Allergic airway inflammation is not affected in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice

Inflammatory cells are recruited to the lungs as a consequence of allergic sensitisation and challenge. The total number of cells in the BAL fluid of OVA treated SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice did not differ from the cell number

recovered from IL-4Rα$^{-/lox}$ mice but was significantly higher than the cell number in the BAL fluid of IL-4Rα$^{-/-}$ mice (Fig 4A). To quantify the number of eosinophils in the lung tissue the amount of eosinophil peroxidase (EPO), which is stored in intracellular vesicles in eosinophils until activation, was measured in lung tissue lysate. There was no significant difference in the amount of EPO in the lung tissue between SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ mice (Fig 4B). In IL-4Rα$^{-/-}$ mice, the concentration of EPO was significantly reduced compared to SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice but was higher than in PBS treated control mice. During allergic airway inflammation the lung tissue and airway smooth muscle cells are infiltrated by mast cells. The amount of mMCP-1, a marker for mast cell degranulation, was increased in the lung tissue after challenge with ovalbumin with no significant differences in all mouse strains (Fig. 4C).

It has been shown that after *in vitro* stimulation with IL-13, airway smooth muscle cells release eotaxin, a chemoattractant for eosinophils. Levels of this chemokine were measured in the serum of OVA sensitised and challenged SM-MHC^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice. Eotaxin concentrations were similar in both SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice but significantly lower in IL-4Rα$^{-/-}$ mice (Fig. 4D).

These results show that SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice are not protected from allergic airway inflammation as is the case with IL-4Rα$^{-/-}$ mice, which showed no signs of eosinophilic cell infiltration in the lungs.

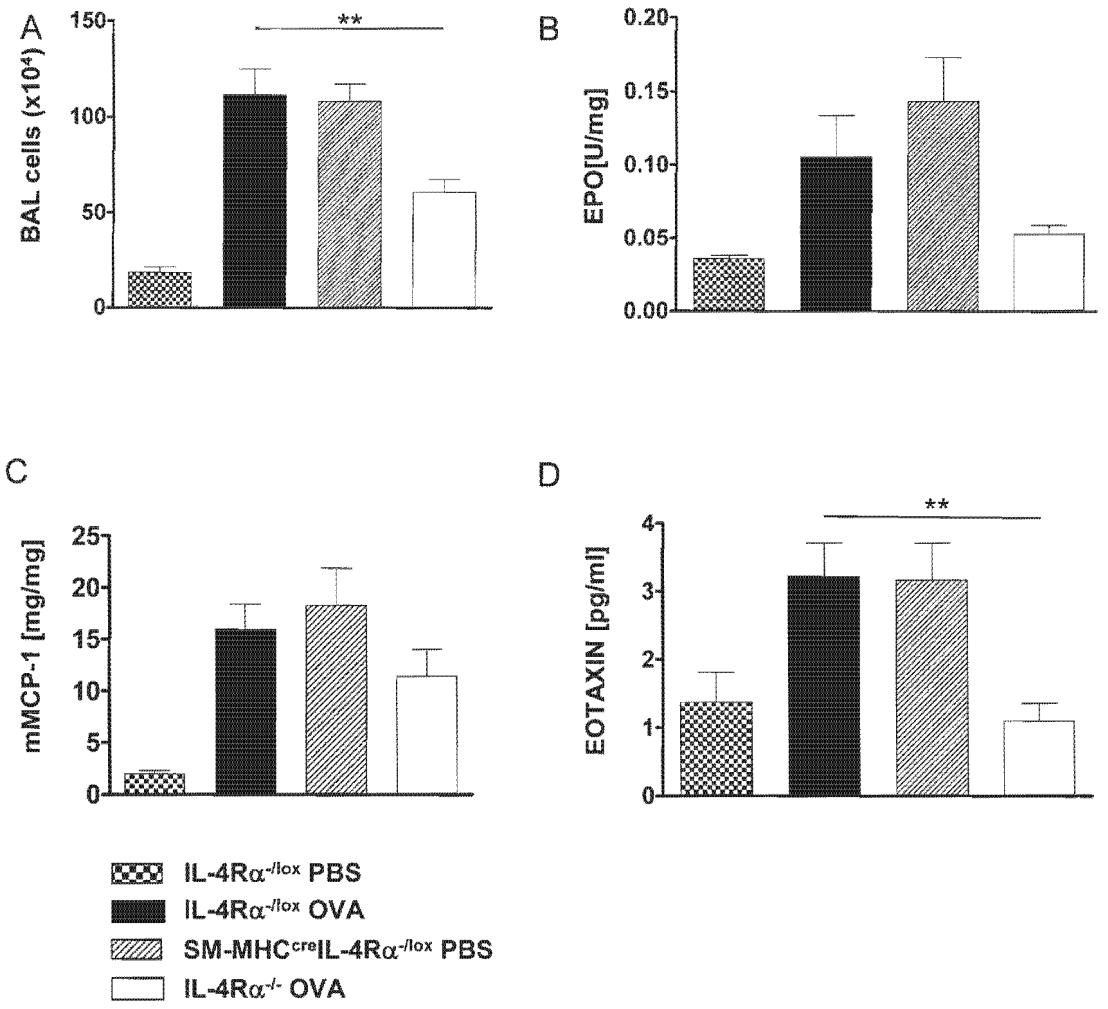

Figure 4. Markers of the lung inflammation are not affected by loss of the IL-4Rα on smooth muscle cells after sensitisation and challenge with OVA. Loss of the IL-4Rα on smooth muscle cells did not alter inflammatory response compared to control mice. Total cell number was counted in BAL fluid (A). Eosinophil peroxidase (B) and mMCP-1 (C) levels were measured in lung tissue lysate. Eotaxin concentration was measured in the blood serum by ELISA (D). Values represent pooled data with SD from two experiments with 4-6 mice per group. (**, $p < 0.005$)

3.4.5 Th2 type cytokine and antibody responses are unaffected in SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ mice

To determine whether T cell dependent immune responses were affected by disrupted smooth muscle cell IL-4Rα, splenocytes and lymphocytes from lung draining mediastinal lymph nodes were stimulated *in vitro* with anti CD3 antibody after sensitization and challenge of SM-MHC^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice with OVA. SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice showed equivalent Th2 type cytokine responses with higher concentrations of

IL-5, IL-9 and IL-13 compared to the IL-4Rα$^{-/-}$ mice. There was no significant difference in the cytokine levels between the SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice (Fig. 5).

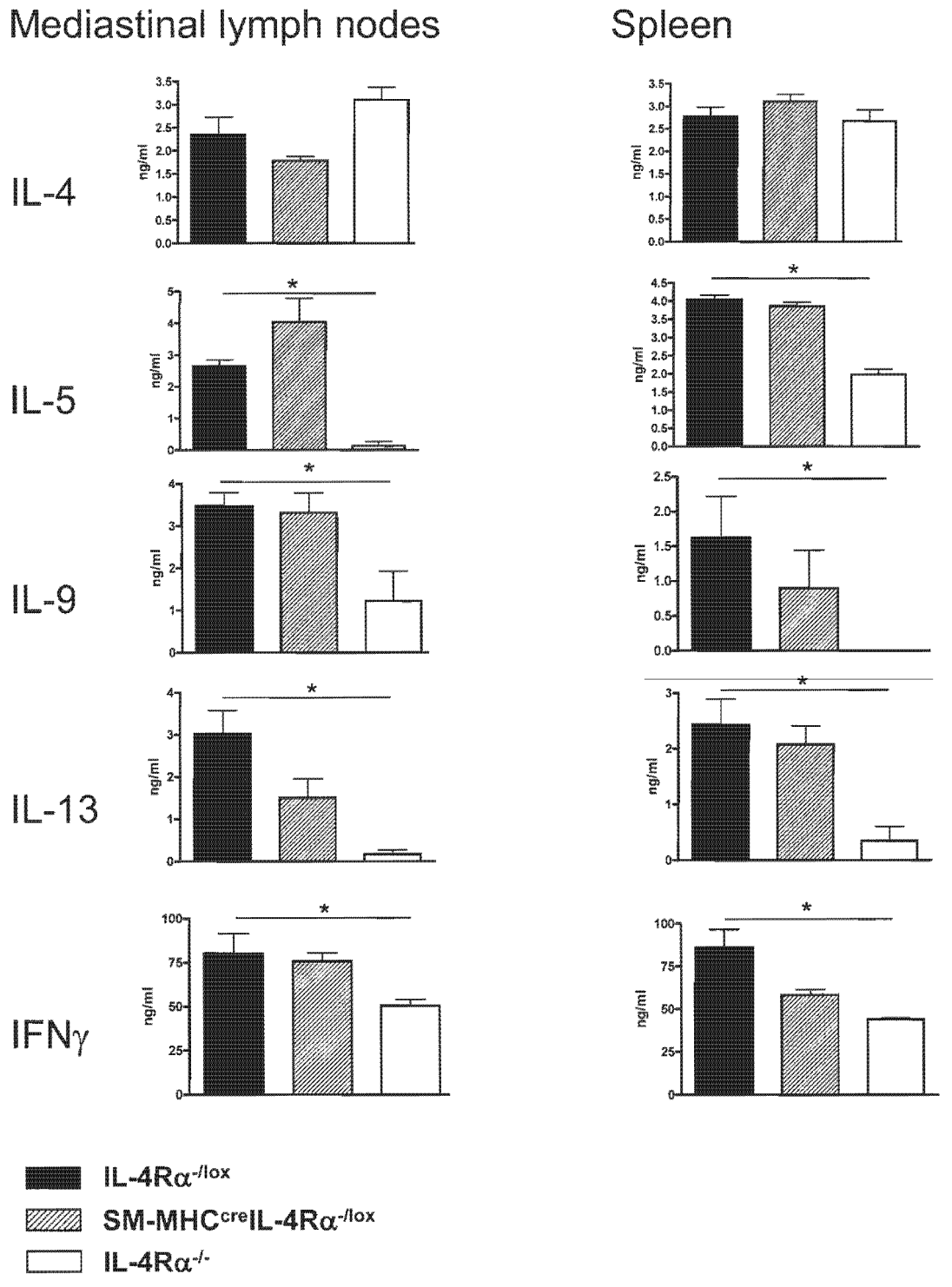

Figure 5. Sensitisation and challenge with OVA induces a Th2 type immune response in IL-4Rα$^{-/lox}$ and SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice. Lymphocytes from mediastinal lymph nodes and splenocytes were restimulated *in vitro* with anti-CD3 antibody and cytokine concentrations were measured in the supernatant. (*, $p < 0.05$)

To investigate whether antibody responses to allergens are affected by IL-4Rα signalling on smooth muscle cells, specific antibody levels in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice were compared to those of IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice after sensitisation and challenge with OVA. Compared to alum treated control groups, serum levels of OVA-specific IgG1 and IgE, were significantly increased in IL-4Rα$^{-/lox}$ and in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice with no significant differences between the two mouse strains (Fig. 6A, B). As expected IL-4Rα$^{-/-}$ mice showed significantly increased levels of specific IgG2a and IgG2b (Fig. 6C, D) but impaired OVA specific IgE and IgG1 antibody titres.[10]

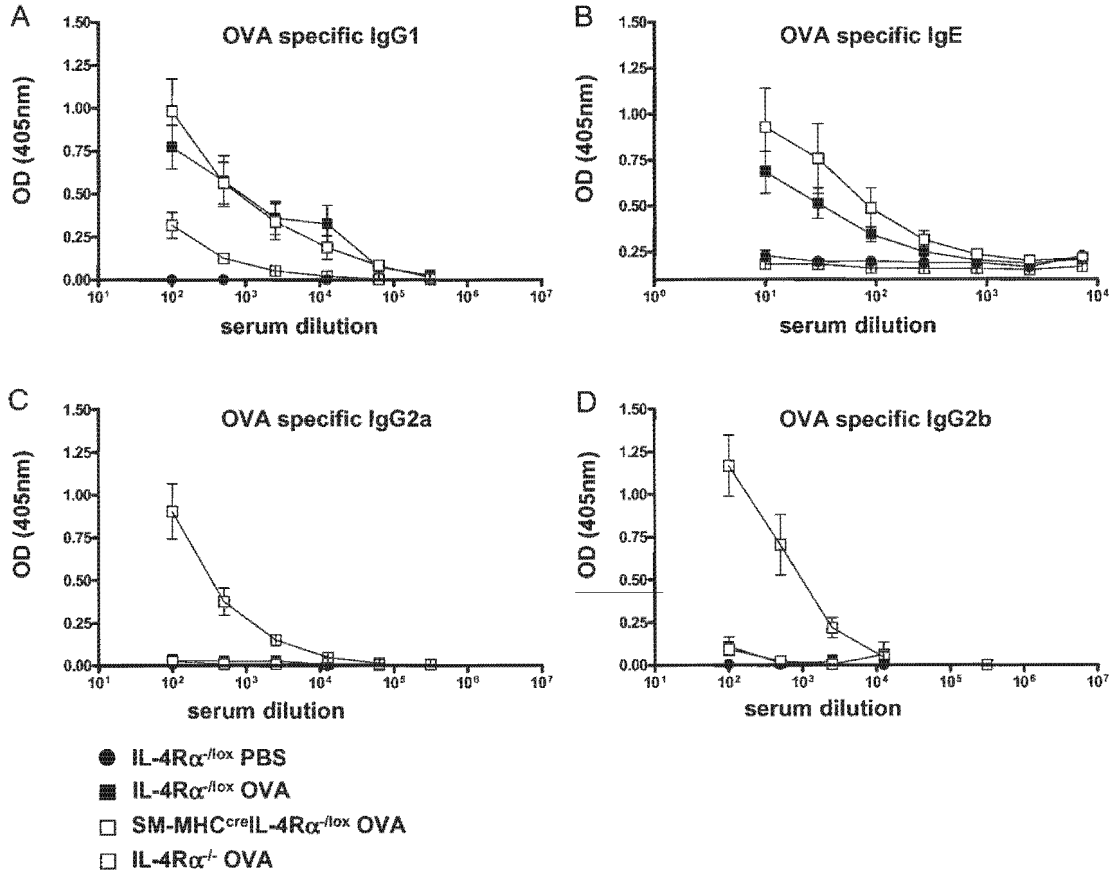

Figure 6. The allergen specific antibody response of SM-MHCcreIL-4Rα$^{-/lox}$ mice resembles the antibody response of IL-4Rα$^{-/lox}$ mice. OVA specific antibodies were measured by endpoint titration ELISA. Tested subclasses were IgG1 (A), IgE (B), IgG2a (C) and IgG2b (D). Data represent pooled values with SD from two experiments with 4-6 mice per group.

3.5 Discussion

To investigate the contribution of smooth muscle cell IL-4 and IL-13 signalling in the onset of allergic asthma *in vivo*, SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice were used, which have a cell specific disruption of IL-4Rα on smooth muscle cells. Comparison of these mice to IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice showed that impaired IL-4Rα signalling in smooth muscle cells had no effect on the major aetiological markers of allergic asthma.

The demonstration of *il4ra* gene disruption in ASMC of SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice in this study confirmed the integrity of this transgenic mouse. As expected the exon 8 to exon 5 ratio was significantly reduced in the trachea of SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice. Furthermore, enrichment of smooth muscle cells by cell sorting of α-actin positive cells from the lung further reduced the exon 8 to exon 5 ratio. The proportion of α-actin negative cells in the purified smooth muscle cell population was 30%. This fact rather than inefficient disruption of IL-4Rα expression might explain why exon 8 copies were still detected in SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice. Cre-mediated cell specific gene disruption has been shown to be highly efficient in hemizygous IL-4Rα$^{-/lox}$ mice. In CD4^{+} T cell specific IL-4Rα Lck^{cre} IL-4Ra $^{-/lox}$ mice, the efficiency of gene disruption was 95.48%.[30] As a small number of ASMC might express functional IL-4Rα we tested SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice in a disease model where IL-4Rα expression is a key mediator of the pathology.[31] Following *N. brasiliensis* infection, mucus production in the airways of both IL-4Rα$^{-/-}$ mice and SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice was noticeably reduced. This phenotype is in agreement with previously published data where *N. brasiliensis* induced intestinal mucus production was also reduced in SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice. [14] In conclusion, these data provided convincing evidence that SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice have a smooth muscle cell specific disruption of the *il4ra* gene in the airways.

Both SM-MHC^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice developed AHR after sensitisation and challenge with OVA, and equivalent responses to acetylcholine were seen in both groups. Furthermore, comparable levels of airway inflammation were observed in SM-MHC^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice following treatment with allergen. No difference in concentration of eotaxin in blood serum or eosinophilic inflammation of lung tissue was seen between SM-MHC^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice. As expected, IL-4Rα$^{-/-}$ mice failed to generate a hyperplasic goblet cell mucus response, however both SM-MHC^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice showed hyperplasic goblet cell driven mucus responses. Finally lymphocytes from SM-MHC^{Cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice produced IL-4, IL-5, IL-9 and IL-13 in comparable amounts after *in vitro* stimulation and both mouse strains showed a Th2 type antibody response typical of allergic reactions.

A recent study demonstrated that IL-4Rα signalling in epithelial cells is required for enhanced mucus production in response to allergens. Using mice with disrupted expression of IL-4Rα on clara cells (goblet cell precursors), it was possible to demonstrate a cell specific requirement for IL-4Rα for allergen induced mucus production in a non-immune cell type.[11] Although epithelial cell IL-4Rα signalling was required for mucus production, it was not required for AHR.

The finding that loss of IL-4Rα on smooth muscle cells had no effect on any of the major aetiological markers of allergic asthma is surprising. A large body of *in vitro* data indicated a contribution of smooth muscle cell IL-4Rα in allergic asthma. Several studies showed enhanced responses of cultured murine smooth muscle cells to contractile agonists after treatment with IL-4 and IL-13.[12, 13, 32] In human smooth muscle cells, IL-13 increased intracellular Ca^{2+} responses induced by contractile agonists [12] and decreased responsiveness to bronchodilators.[33] Based on these results it was believed that narrowing of the airways in allergic asthma was influenced by direct effects of IL-4 and IL-13 on ASMC. However, it was shown here that AHR in this model was independent of IL-4Rα signals in ASMC, indicating that enhanced responses

to contractile agonists after treatment with IL-4 or IL-13 observed *in vitro* may be of little or no relevance to AHR development *in vivo*. Furthermore, it was shown that IL-4Rα signalling on smooth muscle cells is not essential for inflammatory responses and eotaxin release during acute phase allergic asthma. As lung endothelial cells express eotaxin in response to IL-13 [34] in a STAT6 dependent manner [35] these cells may be responsible for the IL-4Rα dependent increases in serum eotaxin observed here contributing to the airway inflammation. On the other hand we have previously shown that direct effects of IL-13 on airway epithelial cells did not induce airway inflammation *in vivo*.[36]

Using the SM-MHC^{Cre}IL-4Rα$^{-/lox}$ mice enabled us, for the first time, to test *in vivo* the assumptions about the role of IL-4Rα on ASMC derived from *in vitro* studies. No evidence was found to support an important role for smooth muscle cell IL-4Rα in allergic asthma. The presence of IL-4Rα on ASMC in allergic asthma was not, as previously suggested, critical for the disease outcome, highlighting the importance of *in vivo* studies of complex diseases such as allergic asthma which are characterised by complicated interactions of different cell populations. However, ASMC do have the ability to produce a wide range of cytokines and chemokines upon stimulation [37] which are likely to play an important immunoregulatory role in allergic asthma independent of direct responsiveness to IL-4Rα. Such candidates are likely to define the role of ASMCs in allergic asthma.

The results presented here were derived from a mouse model of allergic asthma but the situation is likely to be similar in the human disease. As such the data presented here has important consequences for the development of new treatments of allergic asthma and therapies targeting IL-4Rα in the lung might have no direct effect on ASMC.

3.6 References

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Chapter 4

Impaired Alternative Macrophages Activation does not affect the Development of Experimental Allergic Asthma

4 Impaired Alternative Macrophages Activation does not affect the Development of Experimental Allergic Asthma

4.1 Summary

Background

Development of alternatively activated macrophages (AAM) is induced by the Th2 cytokines IL-4 and IL-13 via the IL-4Rα. AAM have been associated with allergic asthma and parasite infections in clinical and experimental investigations but their role in the development of the disease is not defined. Therefore a model of acute allergic asthma was used to compare mice deficient in AAM with control mice. The contribution of AAM in experimental asthma was investigated using $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice which have a macrophage/neutrophil specific disruption of the *il4ra* gene. These mice were compared to IL-4Rα deficient ($IL\text{-}4R\alpha^{-/-}$) and normal control mice ($IL\text{-}4R\alpha^{-/lox}$).

Methods

Allergic asthma was introduced in mice by repeated sensitisation with ovalbumin/ aluminium hydroxide on days 0, 7, 14, followed by intranasal allergen challenge on days 21-23. Mice were investigated for the presence of AAM in the lungs as well as airway hyperresponsiveness, airway inflammation, allergen specific antibody production, Th2 type cytokine responses and lung pathology.

Results

Whereas AAM, identified as Ym1 positive macrophages, were abundant in control mice after allergen challenge, only few were found in $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice. A typical Th2 cytokine response developed in $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice but production of IL-4 and IL-13 in $CD4^+$ T cells was significantly impaired. Both $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ and control $IL\text{-}4R\alpha^{-/lox}$ mice developed all the major signs of allergic asthma. Increased IgE antibody responses, airway

hyperresponsiveness, airway inflammation and mucus production were not different in LysM^{cre}IL-4R$\alpha^{-/lox}$ mice as from controls.

Conclusion

Despite the presence of AAM at early stages of allergic airway inflammation, these cells are not required for the onset of the disease.

4.2 Introduction

Alternatively activated macrophages (AAM) are associated with human[1, 2] and experimental allergic asthma, but their specific role in the pathology of this disease needs to be defined. In the study presented here their role in allergic airway disease was investigated using LysM^{cre}IL-4R$\alpha^{-/lox}$ mice deficient in AAM.[3]

Allergic asthma is a chronic disease of the airways characterised by inflammation of the airways, excessive mucus production in the airway epithelium, airway hyperresponsiveness and airway remodelling. A strong correlation between disease severity and the presence of T helper 2 (Th2) type cytokines, including IL-4, IL-5, IL-9 and IL-13, has been shown in clinical and experimental investigations. Models of experimental asthma highlight the importance of IL-4 and IL-13 and their shared receptor IL-4Rα for the development of main disease symptoms. Whereas IL-4 seems to be important for the initiation of the Th2 type immune response driving allergic asthma,[4] some of the main symptoms are dependent on IL-13.[5] Mice deficient for IL-4Rα show abrogation of airway hyperresponsiveness, hyperplasia of mucus-producing goblet cells, allergen specific IgE and eosinophilic airway inflammation.[6] This shows the general importance of the IL-4Rα for the development of allergic symptoms in experimental asthma, but the cell specific contributions of IL-4Rα signalling need to be identified.

IL-4Rα responsive immune cells present in the allergic lung are involved in the development of asthmatic symptoms. Experimental allergic asthma is critically dependent on CD4$^+$ T cells. Allergen specific IgE, critical for atopic

sensitisation, is produced by B cells in an IL-4 dependent manner.[7] Mast cells, basophils and eosinophils contribute to disease development by the release of inflammatory mediators like histamine or leukotrienes and may be important sources of IL-4 and IL-13.[8, 9]

The most abundant immune cells in the healthy lung are macrophages residing in the alveolar spaces of the airways.[10, 11] Along with epithelial cells [12] macrophages control the innate immune defence in the airways and express a variety of pattern recognition receptors like mannose receptors or β-glucan receptors. Under disease conditions blood monocytes are recruited to the lung and differentiate into lung macrophages.[13] In asthmatic patients [14-16] and in animal models of allergic asthma [17, 18] numbers of airway macrophages are increased. Such data suggest that macrophages play an important role in allergic inflammation of the lung, but their precise role needs to be defined. Experimental data is conflicting, demonstrating both suppression [19-22] and induction of allergic symptoms in mice.[23] Despite the conflicting results, macrophages might play an important role in regulating inflammatory responses in the lung.

Depending on the local cytokine environment, activated macrophages develop into different subsets. Th1 cytokines, especially IFN-γ, induce classical activation of macrophages which is characterised by secretion of proinflammatory cytokines and upregulation of inducible nitric oxide synthase (iNOS), resulting in nitric oxide (NO) production by hydrolysis of L-arginine. These so called classically activated macrophages (CAM) have antimicrobial, phagocytotic and proinflammatory functions.

The Th2 cytokines IL-4 and IL-13 induce the development of alternatively activated macrophages (AAM) by signalling through IL-4Rα.[13] Alternative activation induces arginase I expression whereas inducible nitric oxide synthase (iNOS) is suppressed.[24] Synthesis of prolines, precursors of collagen, by arginase implicated an important role of AAM in tissue fibrosis and wound healing.[13] AAM are distinct from classically activated macrophages (CAM) by the expression of the surface markers Ym1, [25, 26] FIZZ1 [27] and mMGL1/2 [28] and upregulation of PPARγ [29] and mannose receptor.[13] Mannose receptor and mMGL1/2 are involved in antigen

recognition and phagocytosis but precise contributions of the specific markers in AAM function and disease development are not known. AAM have been shown to control obesity and insulin resistance in mice and might be beneficial for the prevention of diabetes.[29] Elevated numbers of AAM are associated with several diseases including allergic asthma,[2, 13] parasite infections [30, 31] and lung fibrosis [32, 33] but a defined role in most diseases needs to be identified. Infection studies in mice with the nematode *Heligmosomoides polygyrus* showed that induction of AAM by memory Th2 cells mediated a protective immune response against the parasite.[34] AAM were also essential for granuloma formation and the survival of mice after infection with *Schistosoma mansoni* by downregulating a harmful Th1 immune response.[3] Infection of mice with the nematode *Nippostrongylus brasiliensis* induces a strong Th2 type immune response and leads to the induction of AAM in the lung.[35, 36] In mouse models of allergic asthma, AAM have been induced in the lung after allergen challenge. Development of chronic pulmonary *Cryptococcus neoformans* infection in mice was accompanied with induction of AAM leading to the development of symptoms similar to allergic asthma.[37, 38] Expression of the chitinases Ym1 and 2 in lung macrophages was dependent on IL-4Rα signalling in an OVA model of allergic asthma.[26, 39, 40] In the same model, arginase I expression was upregulated in an IL-4Rα dependent manner [2] and its inhibition by RNA interference attenuated IL-13 induced allergic symptoms.[41] A recent study has shown that IL-13Rα1 signalling is not necessary to induce AAM differentiation in a mouse model of allergic asthma suggesting that IL-4Rα signalling might be sufficient.[42] In addition to the results from mouse models, AAM are also associated with human allergic asthma. Numbers of AAM in atopic patients were increased when compared to healthy control persons [1] and high numbers of arginase expressing macrophages could be found in the bronchalveolar lavage (BAL) from individuals with asthma.[2]

These studies demonstrated an association of AAM with allergic asthma, suggesting an important role for these cells in the development of the disease. Nevertheless any contribution by AAM to the pathology remains to be defined. $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were used in this work to investigate the function of

AAM in the acute phase of ovalbumin induced allergic asthma in mice (Fig 1A). LysM^{cre}IL-4R$\alpha^{-/lox}$ mice have a cell specific disruption of the *il4ra* gene in macrophages and neutrophils (Fig. 1B) and are therefore unable to produce AAM.[3] These mice allowed for the first time a direct investigation of the role of AAM in the development of allergic asthma.

4.3 Materials and Methods

4.3.1 Mice

The generation and characterisation of IL-4R$\alpha^{-/-}$ and LysM^{cre}IL-4R$\alpha^{-/lox}$ mice on a Balb/c genetic background was described previously.[3, 43] Hemizygous IL-4R$\alpha^{-/lox}$ mice were used as wild type control groups in all experiments (Fig. 1A).[3] The experiments were performed with 6-8 weeks old female animals. Mice were housed in the animal unit of the University of Cape Town under specific pathogen free barrier conditions using individually ventilated cages. All experiments were approved by the Research Animal Ethics Committee of the University of Cape Town.

4.3.2 Genotyping

The genotypes of mice were determined by PCR prior to the experiments as described previously [3]. Specific PCR primer pairs were for the IL-4Rα 5'-GTAC AGCGCACATTGTTTTT-3' and 5'-CTCGGCGCACTGACCCATCT-3'; deletion 5'-GGCTGCCCTGGAATAACC-3' and 5'-CCTTTGAGAACTGCGGG CT-3'; loxP 5'-CCCTTCCTGGCCCTGAATTT-3' and 5'-GTTTCCTCCT ACCGCTGATT-3'; and Cre, 5'-ATGCCCAAGAAGAAGAGGAAGGT-3' and 5'-GAAATCAGTGCG TTCGA ACGCTAGA-3'. PCR conditions were as follows: 94°C for 1 min, 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min for 40 cycles on an MJ thermocycler (Biozym Diagnostik, Germany)

4.3.3 Allergen sensitisation and challenge

The allergen sensitisation and challenge protocol to ovalbumin (OVA, grade V, Sigma, Germany) was described previously [18] and used with slight modifications. IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice were sensitised on day 0, 7 and 14 with 50μg OVA in 200μl PBS/ 1.3% aluminium hydroxide (Sigma) by intraperitoneal injection. On day 21, 22 and 23 mice were anaesthetised with 200μl Ketamine (Centaur Labs, South Africa)/ Xylazine (Bayer, South Africa) and challenged with 0.1mg OVA in 50μl PBS by intranasal administration. PBS control groups were treated identically except OVA was missing in the solutions. Airway hyperresponsiveness was measured on day 24 and mice were killed and analysed, on day 25 (Fig. 1B).

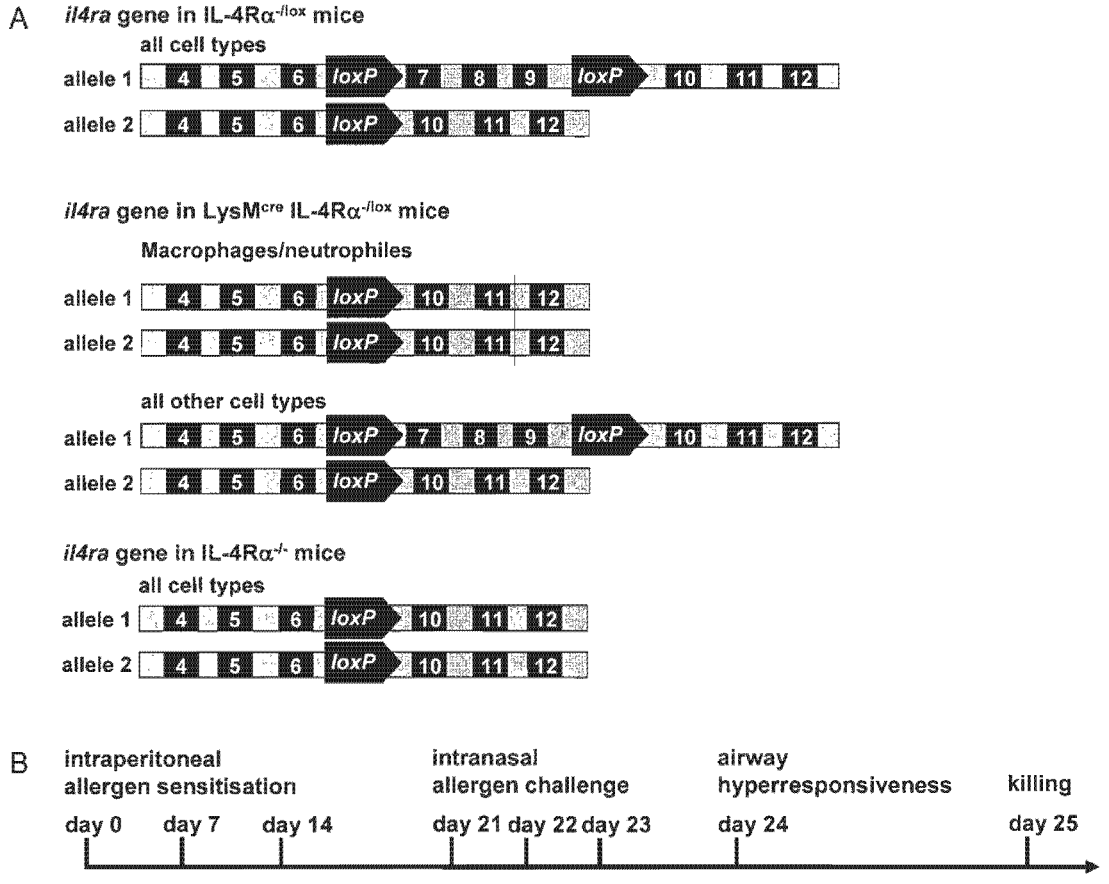

Figure 1. Experimental model of ovalbumin induced allergic airway disease in LysM^{cre} IL-4Rα$^{-/lox}$ mice. Black boxes represent exons of the *il4ra* gene locus in IL-4Rα$^{-/lox}$, LysM^{cre} IL-4Rα$^{-/lox}$ and IL4Rα$^{-/-}$ mice (A). Mice were sensitised intraperitoneally (i.p.) with 50μg ovalbumin (OVA) in 200μl PBS/ alum on day 0, 7 and 14. This was followed by 3 intranasal (i.n.) challenges of anesthetized mice with 0.1mg OVA in 50μl PBS. The PBS control group was treated identically except antigens were absent from the solutions (B).

4.3.4 Airway Hyperresponsiveness

Responsiveness to β-methacholine (Sigma) was assessed in conscious unrestrained mice using a whole body plethysmograph (emka Technologies, France) and analysed with iox2 software (emka Technologies, France). This system measures pressure changes induced by the breathing of the mice and computes enhanced pause (Penh) values, a dimensionless parameter calculated by the formula

Penh= (expiratory time / relaxation time-1)
x (peak expiratory flow / peak inspiratory flow) [44]

Increasing duration of the late phase of expiration relative to its early phase and increasing pressure change during expiration relative to the pressure change during inspiration results in increasing Penh values [45]. It has been shown previously that changes in Penh values correlate with changes in airway resistance in the Balb/c mouse strain under similar conditions to those used in the present study.[44, 46, 47] Individual mice were placed in plethysmograph chambers and baseline readings were measured for 5 minutes before each exposure to increasing doses of aerosolised methacholine (0, 3.125, 6.25, 12.5, 25, 50 mg/ml methacholine in PBS). The response was measured for 15min after each nebulisation. Results are computed as the average of first 5 minutes minus the average of baseline reading.

4.3.5 Serum antibodies

Blood samples were taken by tail vein bleeding or directly from mice after killing and collected in plasma separator tubes (Microtainer™ SST, BD, USA). Samples were centrifuged for 20min at 6000rcf and stored at -80°C. OVA specific antibodies were measured in blood serum by endpoint titration ELISA. For antigen specific antibodies, 96 well ELISA plates (Maxisorp, Nunc, Denmark) were coated overnight with 50µl OVA in PBS (5µg/ml for IgG1,

IgG2a, IgG2b or 1mg/ml for IgE). For total IgE ELISA, plates were coated with anti-mouse IgE antibody (clone 84.1C, 1/1000 dilution). Plates were blocked for 1h at 37°C with 200µl 2% fat free milk powder in PBS. Serial dilutions of serum samples in 50µl PBS/ 0.1% BSA were added to the wells and plates were incubated overnight at 4 °C. Purified recombinant mouse IgE (BD, USA) was used as a standard for total IgE ELISA, at a starting concentration of 1µg/ml. 50µl alkaline phosphatase conjugated goat anti-mouse isotype specific antibodies (Southern Biotechnology, USA) at a 1/1000 dilution in PBS/ 0.1% BSA were added to the wells and the plates were incubated for 3h at 37°C. Subsequently, plates were incubated with 50µl p-nitrophenylphosphate (Fluka, Switzerland) and colour reaction was stopped with 50µl 1M NaOH. Absorption was measured at 405nm with 492nm as reference wave length using a VersaMax plate reader (Molecular devices, USA). Plates were washed 4x with 200µl per well after each incubation step.

4.3.6 Bronchoalveolar Lavage

Mice were killed by CO_2 asphyxiation and lungs were washed once with 1ml PBS through an 18G IV catheter (Braun, Germany). Recovered bronchoalveolar lavage (BAL) fluid (0.5-0.8ml) was centrifuged for 10min at 400rcf and the cell pellet was resuspended in 0.5ml DMEM after lysis of red blood cells. Total cell numbers in the BAL fluid were determined in trypan blue (SIGMA, Germany) stained aliquots using a hematocytometer. A maximum of $2x10^5$ cells in 200µl was centrifuged on a microscope slide at 800rpm for 5min using a Cytospin centrifuge (Shandon, UK). The slides were air-dried and stained with Rapidiff Stain Set (Clinical Diagnostics CC, South Africa). Differential cell counts for eosinophils, neutrophils, lymphocytes and macrophages were made at 400x magnification and at least 100 cells were counted per slide.

4.3.7 Isolation of $CD4^+$ cells

$CD4^+$ cells were purified from lung draining lymph nodes for *in vitro* restimulation experiments [48]. Lymph nodes were removed from the mice and pooled in DMEM/ 2% FCS for each experimental group. Lymph nodes were pressed through a 70µm cell strainer (BD, USA) and cell suspensions were washed once with 10ml DMEM/ 2% FCS. Single cell suspensions were stained with anti-CD8 (clone 53.6.72), anti-CD11b (clone M1/70), anti-GR-1 (clone RB68C5) and anti-B220 (clone RA36B2) antibodies at a concentration of $1x10^7$ cells/ ml. Stained cells were depleted using goat anti-rat IgG Biomag magnetic beads (QIAGEN, Germany) at 1ml per 10^7 cells. $2x10^5$ $CD4^+$ cells per well were restimulated in anti-CD3 (clone 145-2C11, 10mg/ml) coated U-bottom 96-well plates (Nunc, Denmark) for 96h in IMDM / 10% FCS at 37°C. To determine the purity of the sorted cell populations, an aliquot was stained with anti-CD4-biotin (1:320 dilution, clone GK1.5) and Streptavidin-FITC (1:1000, BD, USA) dilution and analysed by FACS (FACS-Calibur, BD, USA).

4.3.8 Cytokine ELISA

Cytokine concentrations in the supernatant of *in vitro* restimulated $CD4^+$ cells were determined by ELISA. Plates (Maxisorp, Nunc, Denmark) were coated overnight with 50µl anti-IL-4 (clone 11B11), anti-IL-5 (BD, USA), anti-IL13 (R&D Systems, USA) or anti-IFNγ (clone AN18KL6) capture antibodies at 4°C. Plates were blocked for 1h at 37°C with 200µl 2% fat free milk powder in PBS. Samples or serial dilutions of recombinant cytokine standards (BD, USA) in 50µl PBS/ 0.1% BSA were added to the wells and plates were incubated overnight at 4 °C. Biotinylated secondary antibodies were added and the plates were incubated for 3h at 37°C followed by horseradish peroxidase (HRP) labelled streptavidin for 1h at 37°C. Subsequently plates were incubated with 50µl TMB Peroxidase Substrate (KPL, USA) and the colour reaction was stopped with 50µl 2M H_2SO_4. Absorption was measured at 450nm with 540nm as reference wavelength using a VersaMax plate reader

(Molecular devices, USA). Plates were washed 4x with 200µl per well after each incubation step.

4.3.9 Lung Histology and Immunohistochemistry

Lung tissue was fixed in 4% Formaldehyde in PBS, embedded in paraffin and cut in 5-7µm sections. Sections were stained with Periodic acid-Schiff reagent (PAS) or haematoxylin and eosin (H&E). For immunohistochemistry, sections were rehydrated and blocked with 1% H_2O_2 in methanol. After antigen retrieval in 10mM citrate buffer pH6, sections were blocked with 0.1% Avidin followed by 0.01% biotin for 15 min. Sections were stained with biotinylated anti-mouse Ym1 antibody for 90 min (1/100 dilution, R&D Systems, USA) and HRP labelled streptavidin for 30 min (1/400 dilution). Stained section were developed with DAB substrate and counterstained with haematoxylin.

4.3.10 Statistical Analysis

Values are given as mean ± SD and significant differences were determined using ANOVA-test (Graphpad Prism™ software). Values $p< 0.05$ were considered significant.

4.4 Results

4.4.1 Ym1 expression in the lung after allergen challenge

$LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice carry a disruption in the *il4ra* gene in macrophages, but not in other cell types. Ym1 is a member of the mammalian chitinase family and its expression is upregulated in alternatively activated macrophages and airway epithelial cells.[27, 40] Tissue sections were stained with anti-Ym1 antibody to identify positive cells in allergic lungs of mice.

Compared to the PBS treated control group (Fig. 2A,E), OVA treatment resulted in highly increased numbers of Ym1 positive alveolar macrophages in control IL-4Rα$^{-/lox}$ mice (Fig. 2B,F). In LysM^{cre}IL-4Rα$^{-/lox}$ mice (Fig. 2C,G) and IL-4Rα$^{-/-}$ mice (Fig. 2D,H) small numbers of Ym1 positive macrophages were found.

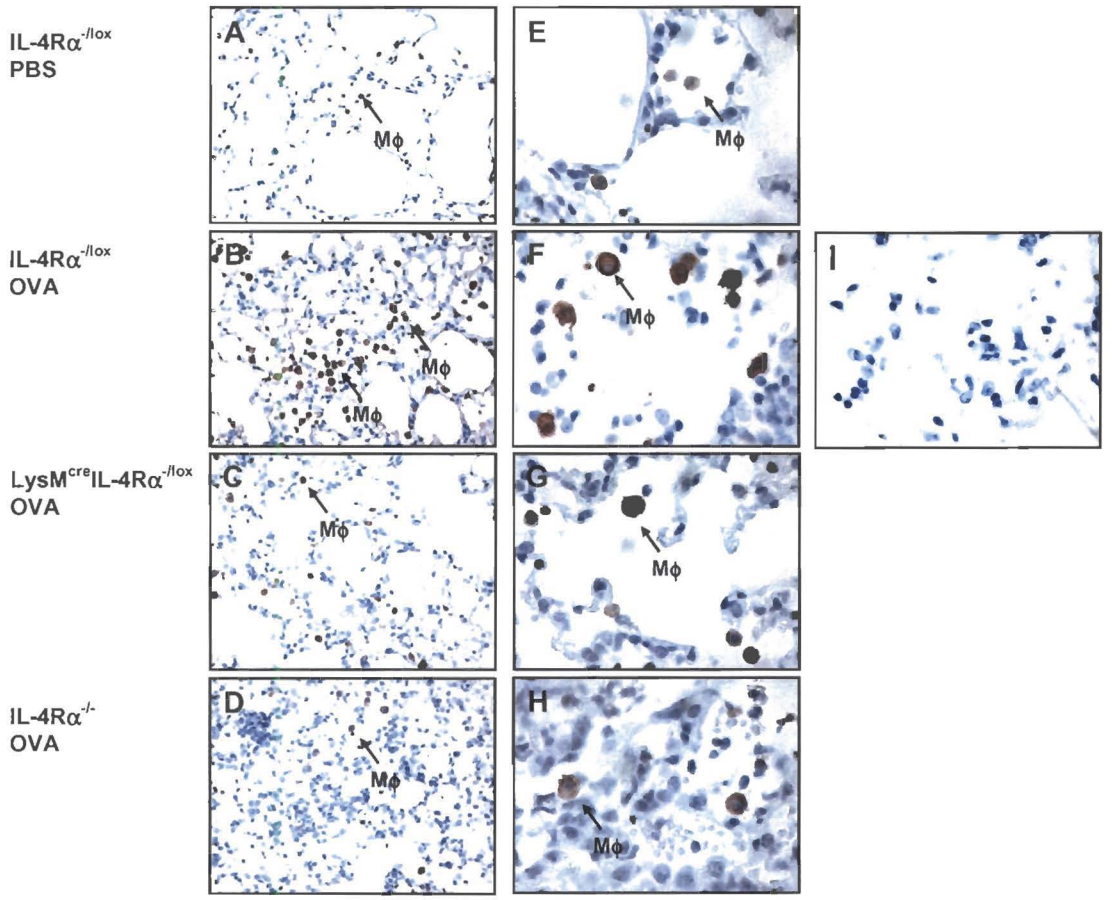

Figure 2. Reduction of Ym1 expression in alveolar macrophages in LysM^{cre} IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice. Lung sections of OVA treated and PBS control mice were stained with anti-Ym1 antibodies to identify Ym1 positive alveolar macrophages. (A-D) 400x magnification, (E-H) 1000x magnification. (I) Isotype control for Ym1 antibody. Mϕ: macrophage. Data are representative of two experiments.

Ym1 was also detected in lung epithelial cells and was upregulated in IL-4Rα$^{-/lox}$ mice after OVA challenge (Fig. 3B), but not after PBS challenge (Fig. 3A). Ym1 expression in epithelial cells was reduced in LysM^{cre}IL-4Rα$^{-/lox}$ mice (Fig. 3C) when compared to IL-4Rα$^{-/lox}$ mice and could not be detected in IL-4Rα$^{-/-}$

mice (Fig. 3D). Negative staining of lung sections from OVA treated IL-4Rα$^{-/lox}$ mice with isotype control antibody (Fig. 2I, 3E) confirmed the specificity of Ym1 staining.

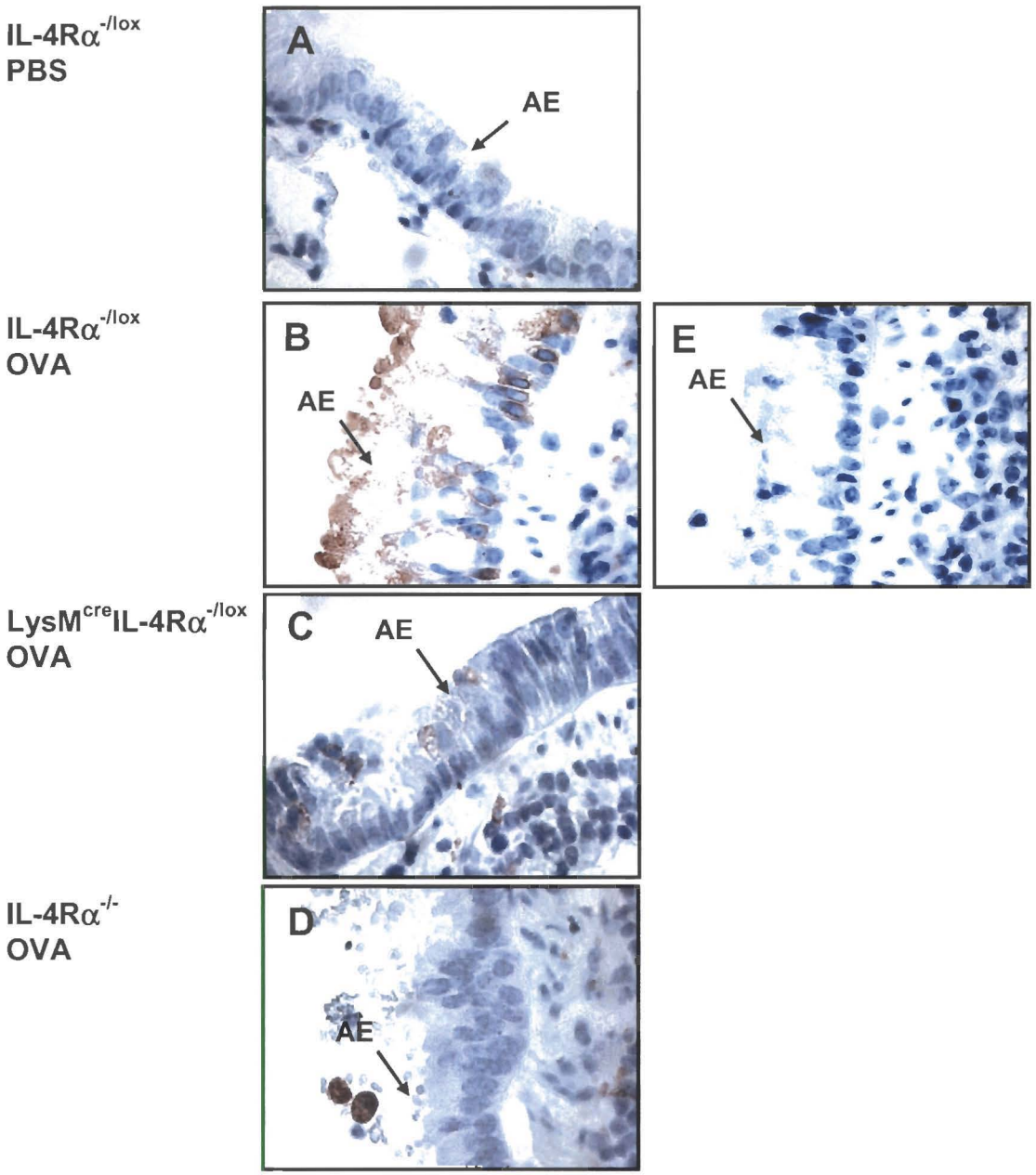

Figure 3. Impaired Ym1 expression in airway epithelium of LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice. Lung sections of OVA treated and PBS control mice were stained with anti-Ym1 antibodies to identify positive airway epithelial cells (A-D). Isotype control for Ym1 antibody (E).1000x magnification. AE: airway epithelium, Data are representative of two experiments.

4.4.2 Cytokine response of $CD4^+$ T cells from lung draining lymph nodes

Development of allergic asthma is driven by Th2 cytokines and Th2 type T cells are considered an important source of IL-4, IL-5 and IL-13. To determine whether T cell dependent immune responses were affected by disrupted IL-4Rα on macrophages, sorted $CD4^+$ T cells from lung draining lymph nodes were stimulated *in vitro* with anti-CD3 antibody after sensitization and after challenge of $LysM^{cre}$IL-$4R\alpha^{-/lox}$, IL-$4R\alpha^{-/lox}$ and IL-$4R\alpha^{-/-}$ mice with OVA. Purity of the sorted $CD4^+$ cell populations was 94-98% as determined by FACS analysis (Fig. 4A). $LysM^{cre}$IL-$4R\alpha^{-/lox}$ and IL-$4R\alpha^{-/lox}$ mice showed a polarised Th2 type cytokine response with elevated concentrations of IL-4, IL-5 and IL-13 when compared to PBS treated IL-$4R\alpha^{-/lox}$ mice and low IFN-γ production. The Th2 type cytokine response from $CD4^+$ cells of $LysM^{cre}$IL-$4R\alpha^{-/lox}$ mice, in particular the production of IL-4 and IL-13, was significantly reduced when compared to IL-$4R\alpha^{-/lox}$ mice. IL-$4R\alpha^{-/-}$ mice showed a Th1 polarized cytokine response reflected in high production of IFNγ and low Th2 cytokine production (Fig. 4B).

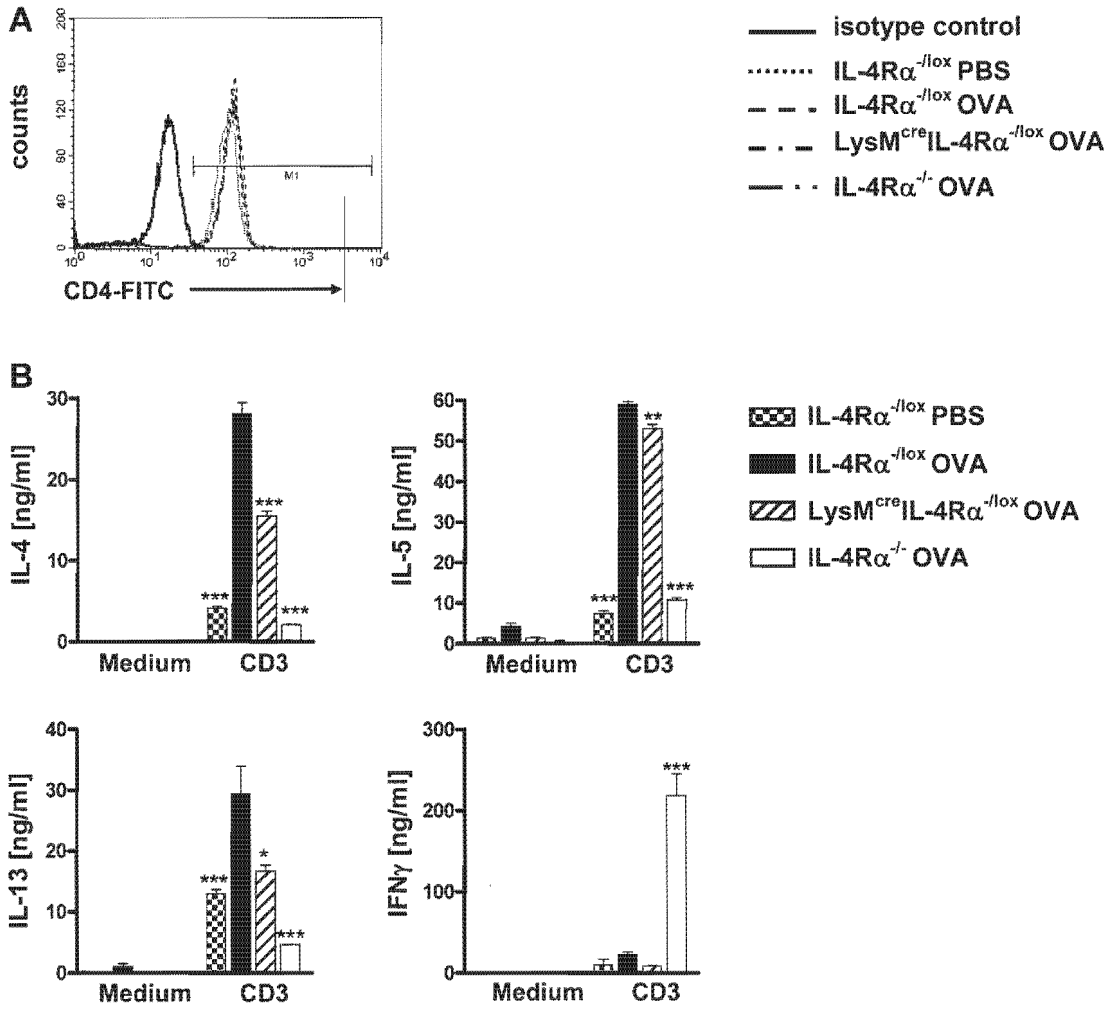

Figure 4. Impaired cytokine production in CD4$^+$ cells from lung draining lymph nodes of LysM^{cre}IL-4Rα$^{-/lox}$ mice. (A) Purity of sorted CD4$^+$ cells was confirmed by anti-CD4 FITC staining and FACS analysis. (B) Cytokine secretion of CD4+ cells from lung draining lymph nodes cultured with anti-CD3 antibodies was measured by IL-4, IL-5, IL-13 and IFN-γ specific ELISA. Data are representative of two experiments ± SD. (**p<0.01; **p<0.01; ***p<0.001 significant differences from OVA sensitised and challenged IL-4Rα$^{-/lox}$ mice)

4.4.3 Airway hyperresponsiveness and airway inflammation

Hyperresponsiveness of the airways to nonspecific bronchoconstrictors is characteristic of allergic asthma. After OVA sensitisation and challenge, mice were exposed to increasing doses of aerosolized β-methacholine and airway hyperresponsiveness was determined by whole body plethysmography. No significant differences were detected between OVA treated LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice. Both groups developed a dose dependent AHR, reflected in increased Penh values. The airway response was significantly elevated

above the PBS treated control group after challenge with 25 and 50mg β-methacholine. As previously shown[7], AHR in OVA treated IL-4Rα$^{-/-}$ mice was abrogated and comparable to the PBS treated IL-4Rα$^{-/lox}$ mice (Fig. 5A).

In allergic asthma, bronchi and alveoli are infiltrated by inflammatory cells with eosinophils being the characteristic cell type. Total numbers of inflammatory cells in the BAL fluid were not significantly different between OVA treated IL-4Rα$^{-/lox}$ (6.4x10^5), LysM^{cre}IL-4Rα$^{-/lox}$ (5.9x10^5) and IL-4Rα$^{-/-}$ (4.3x10^5) mice, but were increased compared to PBS treated IL-4Rα$^{-/lox}$ mice (Fig. 5B). Equal numbers of eosinophils were found in IL-4Rα$^{-/lox}$ and LysM^{Cre}IL-4Rα$^{-/lox}$ mice whereas in IL-4Rα$^{-/-}$ mice eosinophils were significantly reduced (Fig. 5C). No significant differences in numbers of neutrophils, lymphocytes and macrophages were detected between the OVA treated mouse groups.

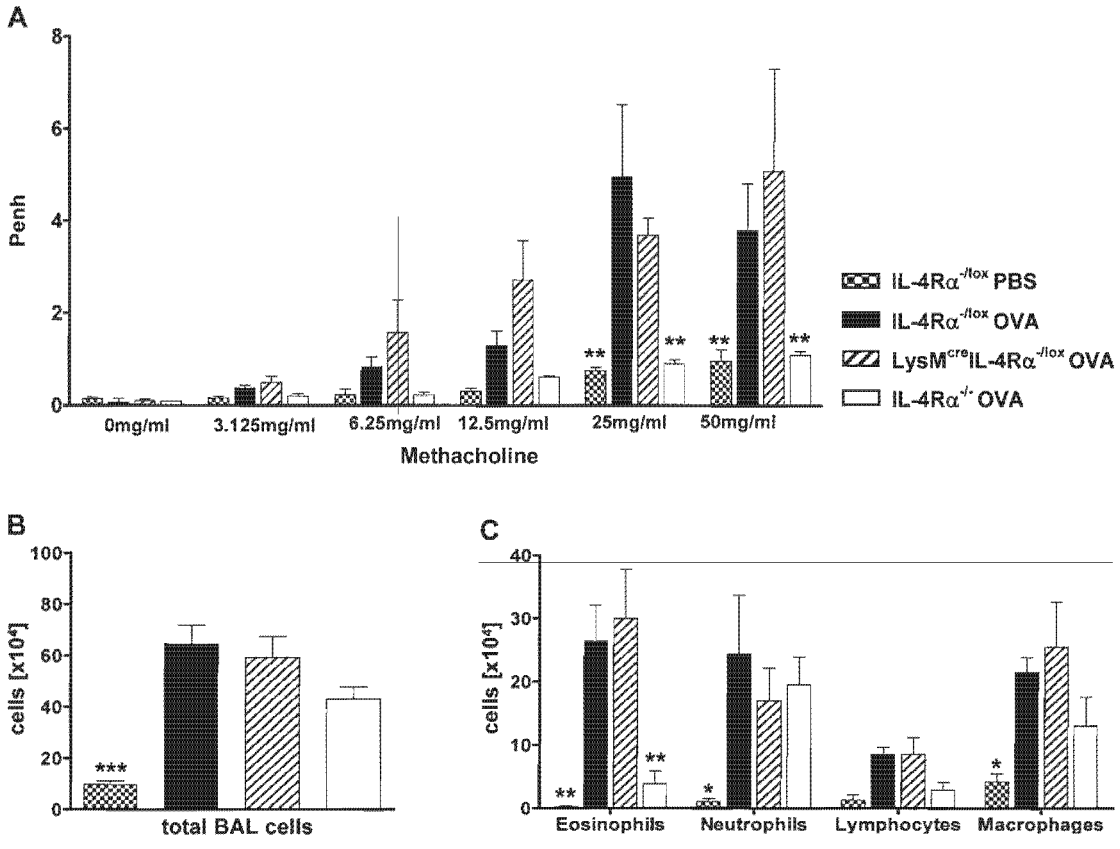

Figure 5. Allergic airway response of LysM^{cre}IL-4Rα$^{-/lox}$ mice is similar to response in IL-4Rα$^{-/lox}$ mice. Airway responsiveness to Methacholine was measured by whole body plethysmography and represented as Penh values (A). Total (B) and differential (C) cell numbers were determined in BAL fluid. Pooled data of two experiments ± SD. (*p<0.05; **p<0.01; ***p<0.001 significant differences from OVA sensitised and challenged IL-4Rα$^{-/lox}$ mice)

4.4.4 Lung histology

During allergic airway reactions, inflammatory cells infiltrate the lung tissue around blood vessels and airways. IL-4Rα$^{-/lox}$ (Fig. 6B), LysM^{cre}IL-4Rα$^{-/lox}$ (Fig. 6C) and IL-4Rα$^{-/-}$ mice (Fig. 6D) showed perivascular and peribronchial inflammation while the alum treated control group failed to demonstrate any infiltration of the lungs by inflammatory cells (Fig.6A). H&E staining of lung sections showed that eosinophils were present in the inflammatory cell infiltrate in both LysM^{cre}IL-4Rα$^{-/lox}$ (Fig. 6G) and IL-4Rα$^{-/lox}$ mice (Fig. 6F), whereas this cell type was absent in IL-4Rα$^{-/-}$ mice (Fig. 6H). Hyperplasia of mucus producing goblet cells in the lungs is a characteristic of allergic asthma. Histological examination of PAS stained lungs of OVA sensitized and challenged LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice showed similar severity of goblet cell hyperplasia (Fig. 6B,C) whereas the alum treated control group and IL-4Ra$^{-/-}$ mice showed no signs of increased mucus production (Fig. 6A,D).

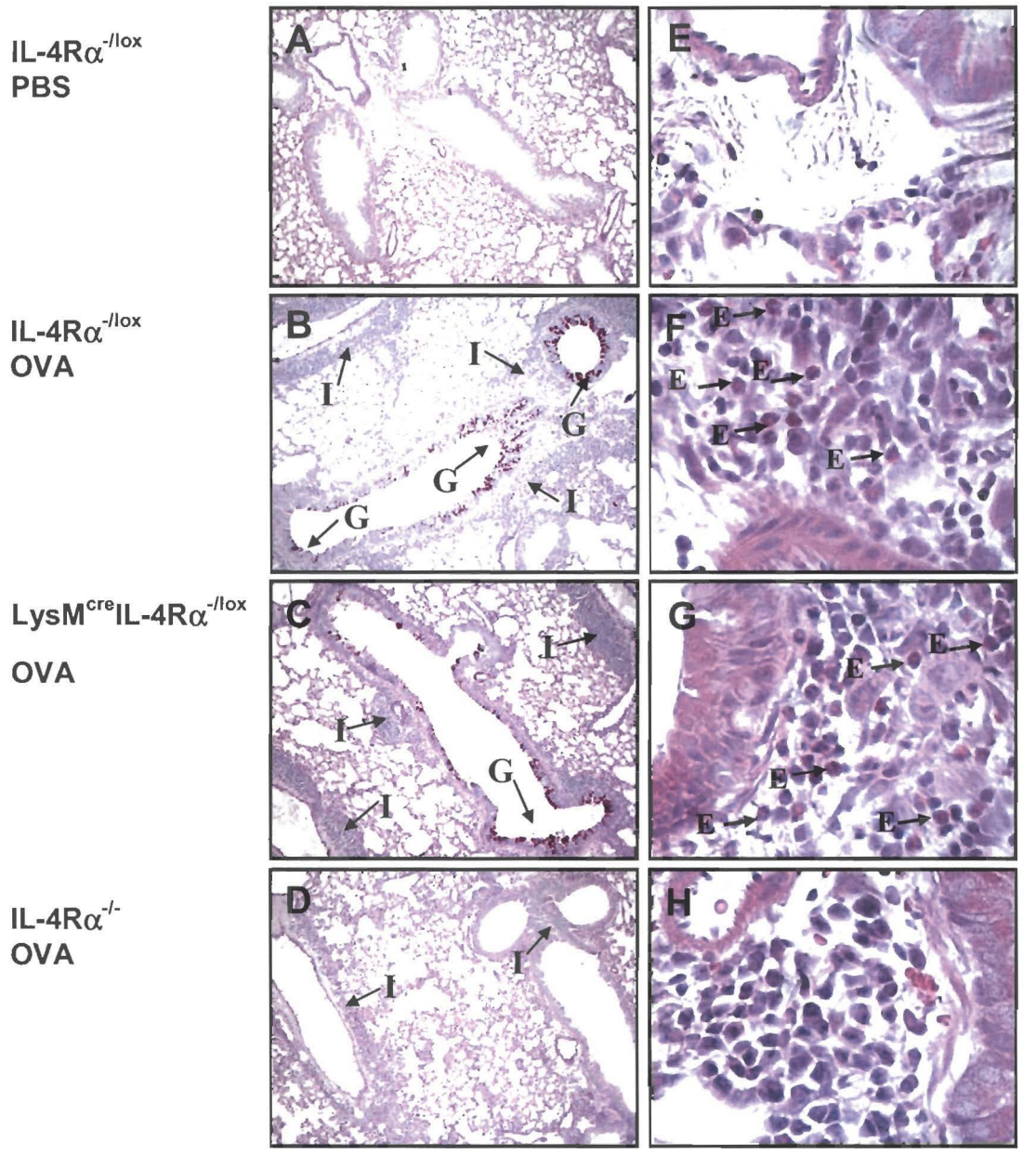

Figure 6. Similar lung pathology in LysM^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ mice after allergen challenge. (A-D) Lung sections of OVA treated and PBS control mice were stained with PAS. G: mucus producting goblet cells; I: inflammatory infiltrate. 100x magnification. (E-H) Lung sections of OVA treated and PBS control mice were stained with H&E. E: eosinophils. 1000x magnification. Data are representative of two experiments.

4.4.5 Antibody response

To investigate whether antibody responses after sensitization and challenge with allergens are affected by IL-4Rα signaling on macrophages, specific antibody levels in IL-4Rα$^{-/lox}$ mice were compared to those of LysM^{cre}IL-4Rα^{-}

$^{/lox}$ and IL-4Rα$^{-/-}$ mice after sensitisation and challenge with OVA. Compared to alum treated control groups, serum levels of OVA specific IgE (Fig. 7A) and IgG1 (Fig. 7C) were increased in IL-4Rα$^{-/lox}$ and in LysM^{cre}IL-4Rα$^{-/lox}$ mice with no significant differences between the two mouse strains. As expected, IL-4Rα$^{-/-}$ mice showed highly increased levels of specific IgG2a and IgG2b (Fig. 7D,E) but impaired OVA specific IgE and IgG1 (Fig. 7A,C) antibody titres.[7] In accordance with the specific antibody response, total IgE concentrations were elevated after allergen sensitisation and challenge in IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice but not in IL-4Rα$^{-/-}$ or PBS treated IL-4Rα$^{-/lox}$ mice (Fig. 7B).

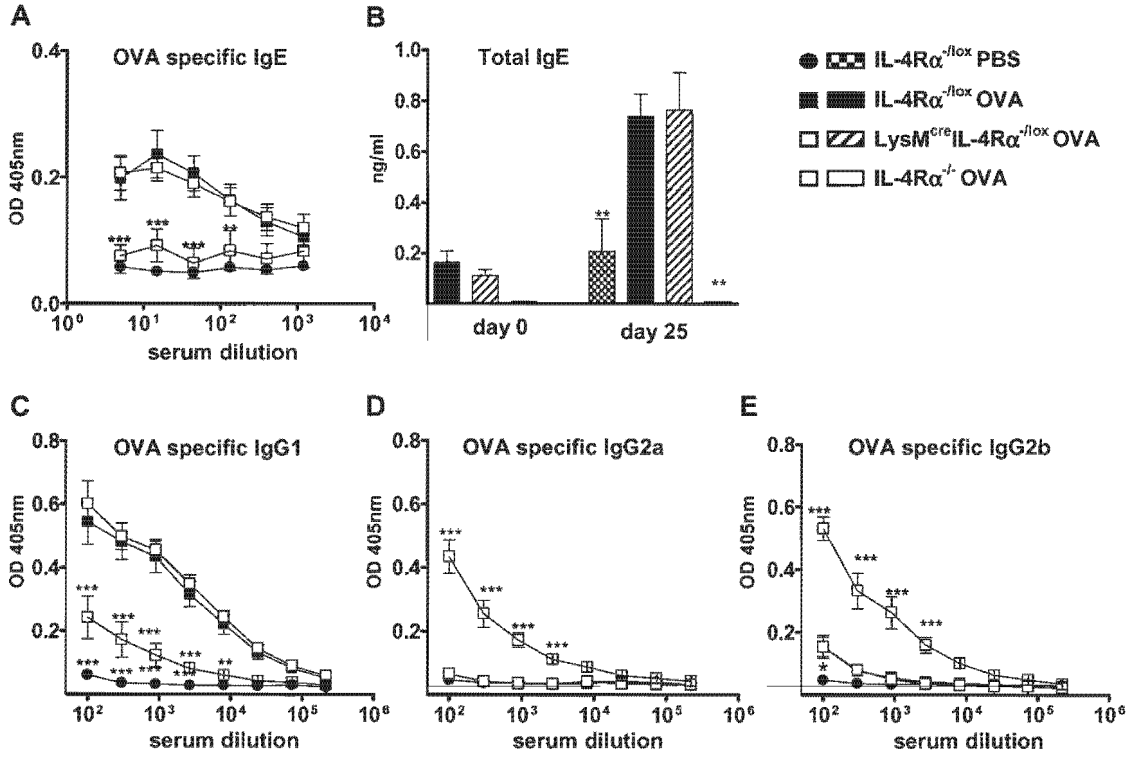

Figure 7. Allergen specific antibody response of LysM^{cre}IL-4Rα$^{-/lox}$ mice resembles antibody response of IL-4Rα$^{-/lox}$ mice. Serum concentrations of antibodies were determined by ELISA. Pooled data of two experiments ± SD. (*** p<0.001; ** p<0.01 significant differences from OVA sensitised and challenged IL-4Rα$^{-/lox}$ mice)

4.5 Discussion

Alternatively activated macrophages have been associated with allergic asthma in a number of studies, but no particular function in the development of the disease has been defined so far.[2, 26, 39-41] The LysM^{cre}IL-4R$\alpha^{-/lox}$ mouse model, which is unable to launch AAM responses, allows the *in vivo* investigation of the role of alternatively activated macrophages in different disease models. Use of these mice has demonstrated important roles for AAM in several studies. For example, it has been shown that survival of *Schistosoma mansoni* infections and the prevention of obesity is dependent on the presence of AAM.[3, 29] Other studies have shown important roles for AAM in memory responses to nematode infections[34] and an association with allergic diseases.[26, 39, 40] Together these results indicated important roles for AAM in Th2 driven pathologies, including allergic asthma. Therefore, LysM^{cre}IL-4R$\alpha^{-/lox}$ mice were used in a model of acute allergic lung inflammation induced by ovalbumin to investigate the cell specific contributions of alternatively activated macrophages to the acute phase of allergic airway inflammation.

This study showed that development of allergic asthma in LysM^{cre}IL-4R$\alpha^{-/lox}$ mice was associated with impaired induction of AAM and significantly reduced cytokine responses in the lung. Despite these effects key pathological markers of ovalbumin induced allergic airway disease remained unaffected.

Impaired AAM development in LysM^{cre}IL-4R$\alpha^{-/lox}$ mice was confirmed by strongly reduced numbers of Ym1 positive macrophages after allergen challenge when compared to IL-4R$\alpha^{-/lox}$ control mice. Macrophage Ym1 expression was not completely abrogated in LysM^{cre}IL-4R$\alpha^{-/lox}$, IL-4R$\alpha^{-/-}$ and PBS control mice, in agreement with previous observations of additional IL-4Rα and Th2-independent control of Ym1 expression in macrophages [36]. Ym1 was also detected in airway epithelial cells of IL-4R$\alpha^{-/lox}$ mice but not IL-4R$\alpha^{-/-}$ mice. Reduced Ym1 expression in airway epithelial cells of LysM^{cre}IL-4R$\alpha^{-/lox}$ mice compared to IL-4R$\alpha^{-/lox}$ mice implicates an important role for IL-4Rα expression in macrophages. Alternative activation of macrophages might result in the production of unknown factors important for the induction of Ym1

in the airway epithelium. IL-13 dependent Ym1 expression in the airway epithelium has been described [40] and the results presented here suggest a new regulatory role for AAM. There is a possibility that IL-4Rα expression in LysM^{cre}IL-4Rα$^{-/lox}$ mice is not only disrupted in macrophages but also in epithelial cells, but this explanation is unlikely as epithelial cell specific disruption of the *il4ra* gene leads to impaired mucus production [17] which was not observed here.

A Th2 type immune response was observed in the lung draining lymph nodes of IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice after allergen challenge. However, in LysM^{cre}IL-4Rα$^{-/lox}$ mice production of the Th2 cytokines IL-4, IL-5 and IL-13 in lung draining lymph nodes was impaired but not directed towards a Th1 type response as IFN-γ production was not elevated as it was in IL-4Rα$^{-/-}$ mice. The reduced Th2 type cytokine response in LysM^{cre}IL-4Rα$^{-/lox}$ mice did not result in the reduction of allergic symptoms and were sufficient for induction of a type 2 antibody response with high levels of IgE and IgG1. Mouse [30, 31] and human [49] AAM are able to inhibit T cell proliferation and activation and downmodulate cytokine responses. By contrast the results presented here show higher cytokine production in the presence of AAM. This might be due to the reduction of Ym1 production in the lung of LysM^{cre}IL-4Rα$^{-/lox}$ mice. Ym1 and other chitinases like AMCase are not only associated with and upregulated during allergic airway responses but have also been shown to positively influence the development of a Th2 immune response.[50]

Despite the strong reduction of AAM numbers in the lung after allergen challenge and an impaired Th2 cytokine response in lung draining lymph nodes, the pathology of allergic asthma was not altered in LysM^{cre}IL-4Rα$^{-/lox}$ mice when compared to IL-4Rα$^{-/lox}$ mice. Allergen specific antibody production was virtually identical in LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice. This suggests that AAM are not critical in the sensitisation phase of allergic responses when a Th2 type memory response against the allergen is established.

Airway hyperresponsiveness was not affected in LysM^{cre}IL-4Rα$^{-/lox}$ mice, demonstrating that IL-4Rα signals in macrophages and neutrophils are not involved here. Interestingly the cellular composition of inflammatory cells in the BAL fluid was not altered in the absence of AAM in LysM^{cre}IL-4Rα$^{-/lox}$

mice. Numbers of neutrophils, macrophages and eosinophils did not differ between LysM^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ mice. Furthermore, eosinophils were present after allergen challenge in the peribronchial inflammatory infiltrate of IL-4Rα$^{-/lox}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice. These results show that AAM are not required for the development of eosinophilic inflammation in allergic asthma. Involvement of AAM in eosinophil recruitment has been suggested in mouse models of nematode infections.[51, 52] These different observations might due to differences between mouse models of nematode infection and allergic asthma. Furthermore, involvement of eosinophil recruitment was only correlated to Ym1 expression in macrophages [51] or demonstrated indirectly in *op/op* mice [52] which show impaired macrophage development. By using LysM^{cre}IL-4Rα$^{-/lox}$ mice the present study showed directly that AAM are not necessary for eosinophil recruitment in allergic asthma.

In summary, the contribution of AAM to the onset of allergic asthma was investigated using LysM^{cre}IL-4Rα$^{-/lox}$ mice with a macrophage/ neutrophil specific disruption of the *il4ra* gene. AAM were present in the early stages of allergic airway inflammation in IL-4Rα$^{-/lox}$ mice but not in LysM^{cre}IL-4Rα$^{-/lox}$ mice. Previous studies have shown that AAM are induced during allergic asthma and the work presented here is in agreement with these observations. However, based on the results presented here this recruitment does not play a role in acute allergic airway pathology. A role for AAM in the chronic phase of allergic asthma has been proposed and future work will aim to define contributions of AAM in the control of chronic allergic airway inflammation.

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Chapter 5

Conclusions and Future Work

5 Conclusions and future work

5.1 Anisakis related allergies

The aim of this study was to compare the impact of two clinically important sensitisation routes to *Anisakis pegreffii*, namely infection with live *Anisakis* worms and sensitisation to *Anisakis* extract by inhalation[1, 2] on the outcome of experimental allergic asthma. We found allergic airway disease to be equivalent in both experimental scenarios and wild type Balb/c mice developed airway hyperresponsiveness (AHR), goblet cell hyperplasia and airway eosinophilia after allergen challenge.

Two findings in this study were of special interest as they were dependent on the sensitisation route.

In wild type mice, sensitisation via the nasal route induced AHR, goblet cell hyperplasia and airway eosinophilia independent of allergen specific antibody responses. However, *Anisakis* specific IgE could not be detected and total IgE levels were not affected by sensitisation. Antigen specific IgG1 was found at low levels compared to *Anisakis* live infection where mice developed a robust allergen specific IgE and IgG1 response.

Development of allergic symptoms in the absence of IgE has been described in mouse and human studies. Whereas IgE was not necessary for the development of allergic symptoms, Th2 cytokines were required in mice[3-5] and elevated in human patients.[6-8] In agreement with these data, sensitisation via the nasal route induced allergen specific Th2 type cytokine responses and development of allergic symptoms in the absence of allergen specific antibodies.

Anisakis infected mice developed IL-4Rα-independent airway hyperresponsiveness in the absence of other typical allergic symptoms such as specific IgE, goblet cell hyperplasia and Th2 type immune responses. Interestingly, IL-4Rα independent AHR was dependent on the route of sensitisation as development of AHR after nasal sensitisation was impaired in IL-4Rα deficient mice. IL-4Rα deficient mice do not develop allergic

symptoms,[9, 10] but development of IL-4Rα independent AHR has been reported.[11] However, IL-4Rα independent AHR required IL-13 and IL-5 [11] and production of both cytokines was impaired in the lymph nodes of IL-4Rα deficient mice in our study. Other sources of IL-13 might be responsible for the development of IL-4Rα independent AHR after *Anisakis* live infection. A mechanism that does not involve IL-13 is another explanation for the development of AHR in IL-4Rα deficient mice. High numbers of neutrophils in the BAL were found after allergen challenge and neutrophilic asthma has been described in human patients. The relation of neutrophils with clinical asthma pathology in is not understood and a mechanism for disease development is not known.[12]

In both sensitisation protocols, AHR could be induced in the absence of allergen specific IgE. This suggests that non-atopic individuals could also develop *Anisakis* induced airway symptoms. Diagnosis of *Anisakis* allergies often relies on the detection of specific IgE [13, 14] and our results may be important especially for the in the occupational environment where nasal sensitisation plays an important role in disease development [1, 15, 16]. Absence of specific IgE might not be sufficient to rule out *Anisakis* allergy, especially in cases of occupational asthma.

Furthermore, we found several potential *Anisakis pegreffii* allergens. A 35 kDa allergen, identified by Western blot analysis, has been identified previously in our laboratory in human and animal studies (unpublished data). No *Anisakis* allergen of this size is found in the literature or allergen data bases (http://www.allergen.org) but *Anisakis* allergens are described for the species *A.simplex*. This 35 kDa protein might represent a specific allergen for the species *A.pegreffii*. The identification of a specific allergen may be important for the diagnosis of *Anisakis* allergy in South Africa as this species is commonly found local fish and a significant cause of allergy.

Indeed, in South Africa fish consumption is common what increases risk of developing *Anisakis* related diseases [2] and a high prevalence of *Anisakis* sensitisation is found in fish factory workers.[1] Other nematode infections, in

particular *Ascaris lumbricoides*, are a common problem in South Africa [17] and could be linked to an increased risk of developing allergy to cross-reactive allergens.[18, 19] As cross-reactivity between *Anisakis* and *Ascaris* allergens has been shown [20] it would be important to investigate whether *Ascaris* infection is able to predispose an individual to *Anisakis* induced allergies. In order to develop reliable diagnostic tools, allergens of *Anisakis pegreffii* need to be characterised and are currently under investigation in our department. Further studies are necessary in order to obtain a better picture of the local situation and to develop prevention strategies and public awareness for the problem. It is also important to understand the immunological mechanisms that are responsible for the development of allergic reactions in order to provide effective therapies and diagnostic tools for allergic patients.

5.2 IL-4Rα in the development of allergic asthma

The general importance of IL-4Rα in the development of allergic diseases and in particular allergic asthma has been shown in mouse models of the disease and in human studies.[21-23] However, specific roles for IL-4Rα in the disease especially its role on different cell types is only partially understood. Transgenic mice with cell type specific disruptions of the *il4ra* gene are an excellent tool for addressing this question. Such cell specific models are relatively new, yet have already yielded substantial data of the cell specific contribution of IL-4Rα in experimental allergy models. For example IL-4Rα dependent murine anaphylaxis has been shown to be driven by IL-4Rα expression on $CD4^+$ T cells, but not macrophages.[24] A study using transgenic mice with an epithelial cell specific *il4ra* gene disruption has demonstrated that goblet cell hyperplasia and mucus production to be critically dependent on IL-4Rα expression in these cells in experimental asthma.[25]

The present study expands on this knowledge by demonstrating the role of IL-4Rα expression on macrophages and smooth muscle cells in experimental allergic asthma induced by ovalbumin. An unpublished study in our laboratory

demonstrated that ovalbumin and *Anisakis* protein extract have similar effects on the onset of allergic airway disease when compared in the experimental protocol used in the study presented here. Therefore, results obtained here are also relevant for *Anisakis* induced allergic disease.

Previous studies have demonstrated a critical role of IL-4Rα expression on macrophages [26] and smooth muscle cells [27] in different models of parasite infections. However, in both mouse models we found no significant contribution of IL-4Rα in the onset of experimental allergic airway disease.

Airway hyperresponsiveness (AHR) and airway inflammation are key features of allergic asthma and *in vitro* data have strongly suggested a direct contribution of IL-4Rα signalling on ASMC to these pathologies.[28] *In vitro* stimulation of ASMC with IL-4/IL-13 increased the contractile response [29, 30] and resulted in release of cytokines such as eotaxin,[31, 32] TARC [33] and VEGF.[34] However, the present study showed that AHR and airway inflammation was independent of IL-4Rα signals on ASMC. This is a surprising and significant result which suggests other signals to be responsible for ASMC contraction and recruitment of inflammatory cells in allergic asthma and future research needs to focus on the identification of these signals.

In addition to ASMC, AAM are also associated with the onset of experimental and human allergic asthma.[35] In the study presented here, AAM were abundant in the early stages of allergic airway disease, but no specific role in the acute phase of allergic airway disease could be defined. Important contributions to disease development were expected as AAM express several molecules associated with allergic asthma such as arginase and Ym1. AAM upregulate arginase expression upon activation[35] and inhibition of arginase attenuated IL-13 induced AHR.[36] Expression of chitinases such as AMCase [37] and Ym1 [38] is regulated by IL-13 in macrophages and airway epithelial cells [39] in experimental and human asthma and a regulatory role for Th2 type immune responses has been proposed for these molecules. In the study presented here we found impaired Ym1 expression in macrophages and airway epithelium in $LysM^{cre}$ IL-$4R\alpha^{-/lox}$ mice together with impaired Th2 type cytokine

production in lung draining lymph nodes. However, the development of a Th2 type immune response and asthmatic symptoms was not affected and LysM^{cre} IL-4R$\alpha^{-/lox}$ mice displayed all key features of the disease, although the Th2 type cytokine response was impaired in lung draining lymph nodes. AAM have been associated with recruitment of eosinophils in murine helminth infections.[40] However, in our model of experimental asthma, the development of airway eosinophilia was not dependent on AAM. LysM^{cre}IL-4R$\alpha^{-/lox}$ mice are only deficient in IL-4Rα activated AAM and other types of macrophages with similar properties as are found, such as IL-10 activated macrophages.[35] Furthermore, the *Cre/loxp* system does not provide disruption in 100% of the target cells.[41] Ym1, a marker for AAM, was still expressed in macrophages of LysM^{cre}IL-4R$\alpha^{-/lox}$ mice and these cells might compensate for the loss of IL-4Rα activated macrophages.

This study highlighted the usefulness of cell type specific IL-4Rα deficient mice and *in vivo* disease models for gaining a better understanding of the mechanisms behind allergic airway diseases. Previous studies suggested important IL-4Rα dependent contributions for ASMC [28] as well as AAM [35] to the development of allergic airway disease but these conclusions were derived from *in vitro* experiment with ASMC or from associations of AAM with the disease. Cell type specific IL-4Rα deficient mice allow a direct and convenient investigation of the role of this receptor in the development of allergic diseases and the results presented here demonstrated this clearly.

However, IL-4Rα signal on ASMC and AAM may play an important role in the development of chronic allergic asthma. The present study used mouse models of the acute early phase of the disease and chronic effects were not addressed. Involvement of ASMC and AAM in airway remodelling, an effect of chronic allergic airway disease, has been suggested and IL-4Rα signalling may contribute to tissue fibrosis [35] and proliferation of smooth muscle cells around the airways.[28] Future work will concentrate on mouse models of chronic allergic asthma and are currently under investigation in our department.

Furthermore, the mechanism behind IL-4Rα dependent disease development in the early and chronic phase of allergic airway inflammation will be investigated in additional cell type specific transgenic mice. In particular, cell specific contributions of the IL-4Rα in T cells, B cells and dendritic cells are of interest as these cells are believed to be crucial for induction of Th2 type cytokine responses, allergen specific IgE and IgG production and allergen presentation to T cells.

The present study showed the importance of the route of sensitisation and IL-4Rα in development of allergic diseases. In the past years substantial progress has been made in the understanding of immunological mechanisms behind allergies, but many aspects remain obscure. Research in this exciting field will continue in future and is necessary to provide new therapeutic strategies.

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