

**Immune and allergic responses to *Anisakis pegreffii*,
with focus on the roles of IL-4, IL-13 and the
IL-4 receptor alpha**

Natalie Nieuwenhuizen BSc(Med) Hons



Thesis presented for the Degree of
DOCTOR OF PHILOSOPHY
In the Division of Immunology,
Department of Clinical and Laboratory Science
UNIVERSITY OF CAPE TOWN
February 2007

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DECLARATION

I, Natalie Nieuwenhuizen, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work or any part thereof has been, is or will be submitted for another degree in this or any other university.

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Signed by candidate

Natalie Nieuwenhuizen
February 2007

University of Cape Town

**In loving memory of my grandparents,
Denis and Peggy Walter.**

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Finally, many thanks to my family and friends for their support and encouragement.

ABBREVIATIONS AND SYMBOLS

Ab – antibody

AD – atopic dermatitis

Ag – antigen

Alum – aluminium hydroxide

Ani s 1 – *Anisakis* allergen 1

AP – alkaline phosphatase

APC – antigen presenting cell

APES – 3-aminopropyltriethoxysilane

Arg-1 – arginase-1

BAL – broncheolar lavage

BCA – bicinchoninic acid

BCIP/NBT – 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium

Bla g 2 – cockroach allergen 2

BSA – bovine serum albumin

CD – cytoplasmic domain

CDC – Centre for Disease Control

CI – confidence interval

CLA – cutaneous lymphocyte-associated antigen

CO₂ – carbon dioxide

ConA – concavalin A

Cre – Cre recombinase

CS – contact sensitivity

DC – dendritic cell

ddH₂O – double distilled water

DEPC – diethyl pyrocarbonate

Der p 1 – house dust-mite (*Dermatophagoides pteromyssinus*) allergen 1

DNA – deoxyribonucleic acid

DTH – delayed-type hypersensitivity

DTT – dithiothreitol
ECP – eosinophil cationic protein
ELISA – enzyme-linked immunosorbent assay
ELISPOT – Enzyme-linked immunosorbent spot
EPO – eosinophil peroxidase
ES – excretory-secretory
FACS – fluorescence-activated cell sorting
FcεRI – high affinity IgE receptor
FcγRII – IgG receptor II
FcγRIII – IgG receptor III
Fel d 1 – cat (feline) allergen 1
FDA – Foods and Drugs Administration
Fig. – Figure
g – gram(s)
HDM – house dust-mite
HRP – horseradish peroxidase
H&E – haemotoxylin and eosin
H₂O – water
IDEC – inflammatory dendritic epidermal cell
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
iNOS – inducible nitric oxide synthase
i.p. – intraperitoneal
IRS – insulin receptor substrate
i.v. – intravenous

IVC – individually ventilated cages
JAK – Janus kinase
KO – knockout
L – litre
Lck – lymphocyte specific tyrosine kinase
LT – lymphotoxin
LysM – Lysozyme M
L₁ – first-stage larva
L₂ – second-stage larva
L₃ – third-stage larva
L₄ – fourth stage larva
M – molar
MBP – major basic protein
MHC – major histocompatibility complex
Min – minutes
mg – milligram
mg/ml – milligrams per millilitre
ml – millilitre
mM – millimolar
MMCP-1 – mouse mast cell protease-1
mol – moles
mRNA – messenger RNA
n – number
N – nolar
ng – nanogram
ng/ml – nanograms per millilitre
NK – natural killer
nm – nanometre
NO – nitric oxide

NOS-2 – nitric oxide synthase 2
OCT – oxacalcitriol
OD – optical density
OR – odds ratio
OVA – ovalbumin
P – probability
PAF – platelet-activating factor
PAS – periodic acid-Schiff
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PNP – 4-nitrophenyl phosphate
PRR – pattern-recognition receptor
RANTES – Regulated on Activation, Normal T-cell Expressed and Secreted
RNA – ribonucleic acid
rpm – revolutions per minute
RT-PCR – reverse transcriptase polymerase chain reaction
SA – somatic antigen
SCF – stem cell factor
Sec – seconds
SEM – standard error of the mean
sIL-13R α 2 – soluble IL-13 receptor alpha 1
sIL-4R α – soluble IL-4 receptor alpha 1
sp – species
SPF – specific pathogen free
spp. – species (plural)
SPT – skin prick test
STAT – signal transducer and activator of transcription
STH – soil transmitted helminth
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
Treg – T regulatory cell
UCT – University of Cape Town
U.K. – United Kingdom
U.S.A. – United States of America
WT – wildtype
WHO – World Health Organization
Wk – week
x – times
 α – alpha
 β – beta
 γ – gamma
 γ c – common gamma chain
 μ g – microgram
 μ g/ml – micrograms per millilitre
 μ l – microlitre
 $^{\circ}$ C – degrees celsius
% – percent
< – less than
-/- – knockout

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SUMMARY

Background: The fish-parasitizing nematode *Anisakis pegreffii* induces gastrointestinal disease and allergy when ingested by humans, and can cause occupational allergy in seafood processing workers. The present study examines immune and allergic responses to *A. pegreffii* in wildtype and gene deficient mice, with special focus on interleukin(IL)-4, IL-13, and the IL-4 receptor alpha (IL-4R α).

Methods: Experimental murine models of *Anisakis* infection, *Anisakis*-induced anaphylaxis and *Anisakis*-induced dermatitis were established in order to gain insight into the immune responses generated against *Anisakis* and unravel mechanisms of allergic disease. Mice deficient in IL-4, IL-13, IL4/IL-13 and the IL-4R α were utilized to elucidate the distinct functions of these molecules. In addition, T-cell specific IL-4R α deficient (Lck^{cre}IL-4R α ^{-lox}) and macrophage specific IL-4R α deficient (LysM^{cre}IL-4R α ^{-lox}) IL-4R α deficient mice were used to unravel the cell specific requirements for IL-4R α expression in anaphylaxis and dermatitis.

Results: *Anisakis* infection elicited rapid infiltration of immune cells into the intraperitoneal cavity in all strains, with IL-13 ^{-/-} and IL-4R α ^{-/-} showing the strongest responses. *Anisakis* infection induced a strong Th2/type 2 response in wildtype mice, characteristic of gastrointestinal helminth infections. When challenged orally with *Anisakis* extract, previously infected mice produced a response typical of food allergy, with itching, diarrhoea and airway mucus hypersecretion. In contrast, allergic responses were reduced and abrogated in IL-4 ^{-/-} and IL-4R α ^{-/-} mice, respectively. Oral dosing of mice with *Anisakis* extract resulted in production of the Th2-associated antibodies IgG1 and IgE, but did not elicit strong Th2 responses in the manner of live larvae, and virtually no allergic response was seen after challenge.

In a model using intraperitoneal sensitization and the adjuvant alum, *Anisakis* proteins were able to cause anaphylaxis comparable to that caused by OVA. OVA-induced anaphylaxis was found to be dependent upon the Fc γ RII/III and associated with mast cell degranulation. Specific deletion of IL-4R α on CD4⁺ T cells abrogated anaphylaxis substantially, despite the presence of IgE and Th2 cytokines in these

mice. IFN- γ was found to be crucial for this protection, as its depletion reversed the resistance of this strain.

Epicutaneous sensitization with *Anisakis* larval antigens induced local inflammation, epidermal hyperplasia, Th2 cytokines, and production of anti-*Anisakis* IgE and IgG1. Intravenous challenge of sensitized mice resulted in anaphylactic shock. Skin inflammation was IL-4 independent but IL-13 dependent, while systemic anaphylaxis reactions were predominantly IL-4 mediated. Macrophage/neutrophil specific expression of the IL-4R α was not required for anaphylaxis but exacerbated skin inflammation. CD4⁺ T-cell specific IL-4R α deficient mice experienced partial inhibition of systemic anaphylaxis, despite showing skin inflammation comparable to that of wildtype mice.

Conclusions: *Anisakis* infection strongly predisposes to allergic responses against ingested *Anisakis* proteins and is more effective than oral sensitization with *Anisakis* proteins alone. However, *Anisakis* proteins alone are able to cause allergic reactions, contrary to the suggestion that live infection is required for reactions. IL-4, IL-13 and the IL-4R α play important and distinct roles in *Anisakis* allergy that vary according to the route of sensitization. Studies with cell-specific IL-4R α knockout mice provided deeper insight into the mechanisms of anaphylaxis and atopic eczema. The data suggest that IFN- γ serves an important protective role during the effector phase of systemic anaphylaxis.

THESIS OBJECTIVES

The aim of this study is to investigate the immune responses induced by *Anisakis* infection and to elucidate mechanisms of *Anisakis* allergy. The study focuses on the role of the cytokines IL-4 and IL-13, and their shared receptor subunit, the IL-4 receptor alpha (IL-4R α).

Specifically, we aim to investigate:

- cellular and humoral immune responses induced by *Anisakis*
- the ability of *Anisakis* proteins to sensitize through ingestion and epicutaneous exposure
- the ability of *Anisakis* proteins to cause ingestion-related allergy and anaphylaxis
- the role of IL-4, IL-13 and the IL-4R α in immune responses to *Anisakis*
- the role of IL-4R α expression on CD4⁺ T cells, macrophages and neutrophils in anaphylaxis
- the role of IL-4, IL-13, the IL-4R α and IL-4R α expression on CD4⁺ T cells, macrophages and neutrophils in *Anisakis*-induced dermatitis and associated allergic sensitization

CHAPTER ONE

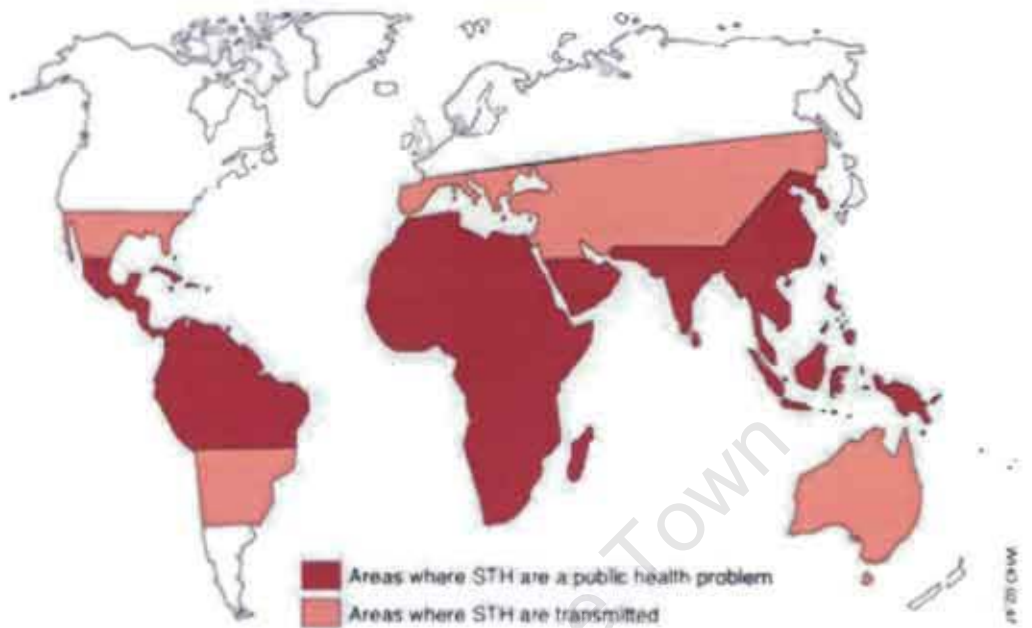
BACKGROUND

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CHAPTER 1: Background

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Global distribution of soil-transmitted helminth infections



Global distribution of schistosomiasis

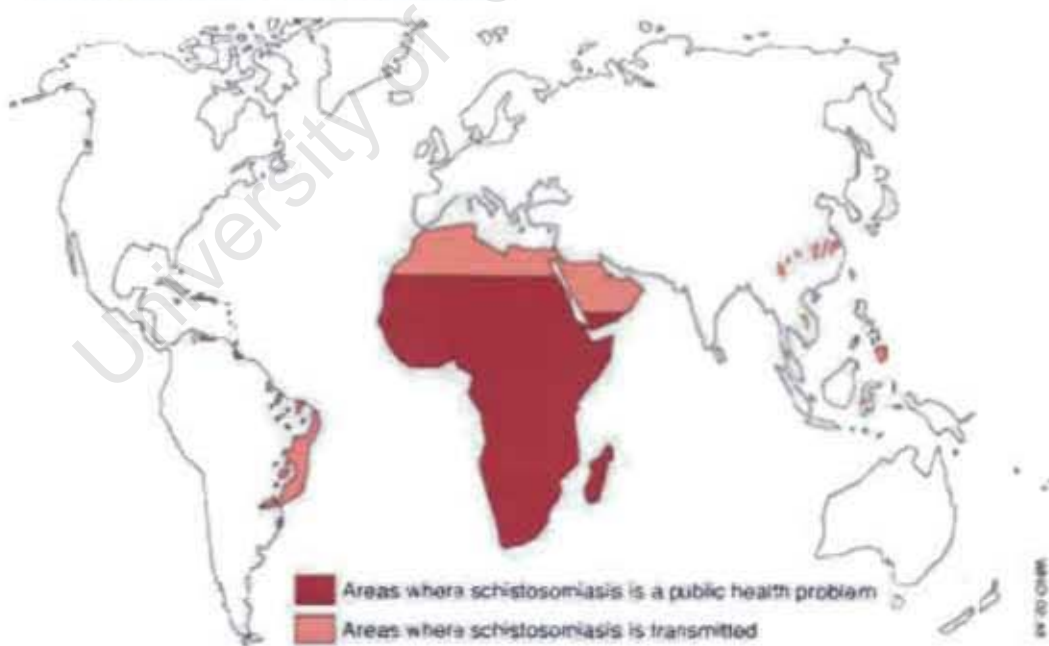


Figure 1.1. Global distribution of soil-transmitted helminth infections and schistosomiasis. Soil transmitted helminths such as roundworms, hookworms and whipworms, as well as the helminths causing bilharzia (*Schistosoma* spp) are more common in the developing world. (Maps from the World Health Organization website¹.)

1.1 Introduction

Infectious and parasitic diseases are the primary causes of death worldwide, despite the fact that most of these diseases are preventable or treatable². One category of parasites – the helminths (parasitic worms) – contributes greatly to the disease burden, especially in the developing world (see Fig. 1.1). Important helminths include *Schistosoma*, the cause of schistosomiasis (bilharzia), and the soil-transmitted helminths (STH), including roundworms, hookworms and whipworms, commonly known as intestinal worms. Around 2 billion people are infected by these helminths, and about 300 million people are severely ill because of them^{2,3}. Schistosomes utilize water snails as a vector, and so schistosomiasis is located around freshwater sources such as lakes and dams in tropical or subtropical regions. STH are more widely distributed and are linked to a lack of access to sanitation; consequently they are more common in areas of poverty^{2,4,5}.

Symptoms of STH infections include nausea, tiredness, loss of appetite and abdominal pain. Most infections are not life-threatening but can cause significant morbidity through sequestering of vitamin A and iron, and by causing loss of appetite^{2,3,5,6}. This can result in growth retardation and impairment of cognitive development^{7,8}. In addition, intestinal worms can suppress immune responses and so may influence the response to other diseases and to vaccines^{9,10}. Heavy infections can cause intestinal obstruction that requires surgery¹¹. Occasionally worms migrate to other organs such as the bile ducts, which also requires surgical intervention¹².

The most common intestinal helminths are the nematodes *Ascaris lumbricoides*, *Necator americanus*, *Trichuris trichuria*, *Ancylostoma duodenalis* and *S. stercoralis*^{4,6,13}. However, occasionally humans may be infected by helminths for which another animal is the natural host. Such infections are termed zoonotic. In 1960 a previously undescribed zoonotic infection was reported by Van Thiel: intestinal infection with the roundworm *Anisakis simplex* in a patient who had eaten raw herring¹⁴ (Fig. 1.2). This case occurred in the Netherlands, but made a huge impact on Japanese parasitologists, who recognized that the Japanese habit of eating raw fish meant that similar cases were highly likely in

their own country¹⁵. Subsequently, in 1964 a special Japanese research group was established to investigate the disease, which was to become known as anisakiasis. Since then, thousands of cases have been reported from Japan and hundreds from European countries where the consumption of fish is high, such as Spain and Italy¹⁶⁻¹⁸. Scattered cases have also been reported from locations world-wide, including other European countries, North and South America, England and New Zealand¹⁹⁻²⁴. It is likely that the rising popularity of sushi and sashimi around the world will increase the incidence of anisakiasis further²⁵. Outside of Japan, Spain and Italy, anisakiasis is highly likely to be misdiagnosed due to a lack of awareness amongst physicians^{18,26}.

1.2 The biology of *Anisakis* species

Anisakis spp. belong to the subfamily Anisakinae, family Anisakidae, superfamily Ascaroidea, suborder Ascaridina, order Ascarida, subclass Secernentrea, Class Nematoda²⁷. Phylogenetic studies indicate that the human parasite to which it is most closely related is *Ascaris* (Fig 1.3)^{28,29}. As a genus, *Anisakis* is found world-wide, but *Anisakis* species are differentially distributed geographically and utilize different host species³⁰⁻³². Recognized species include the three sibling species of the *Anisakis simplex* complex (*A. simplex sensu stricto*, *A. simplex* C and *A. pegreffii*), as well as the morphologically different *A. typica*, *A. ziphidarum*, *A. schupakovi*, *A. physeteris* and *A. brevispiculata*³¹⁻³⁶. These are species that have been confirmed at the genetic level using isozyme analysis or PCR-based approaches. Several other species have been discovered but are not yet properly characterized, such as *A. insignis*, *A. alexandri* and *A. dussumierii*²⁷.

The other genera in the subfamily Anisakinae, collectively known as the anisakids, are *Pseudoterranova*, *Contracaecum* and *Hysterothylacium*. All of these nematodes appear to have similar life-cycles (Fig. 1.4), although their host species vary^{16, 17, 31}. The primary/definitive hosts of anisakids are sea mammals such as whales, dolphins, seals and sea lions, as well as aquatic birds and turtles. *Anisakis* spp utilize cetaceans such as dolphins and whales as primary hosts. Eggs are passed into the sea via the faeces of the cetaceans. The first moult ($L_1 \rightarrow L_2$) takes place inside the egg, releasing free-swimming

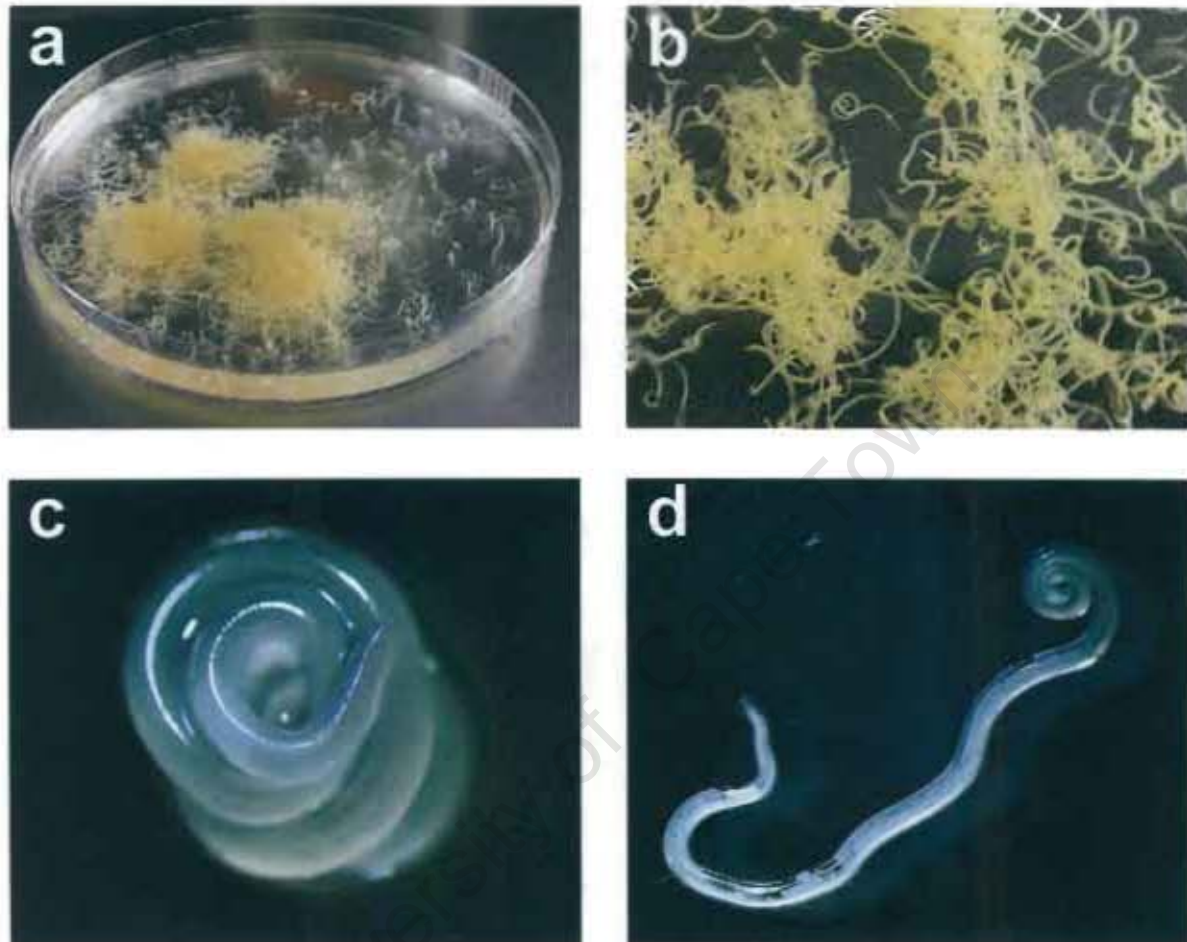


Figure 1.2. *Anisakis pegreffii*. **a)** A large (diameter 13.5cm) petri dish containing *Anisakis pegreffii* third-stage larvae (L₃). **b)** Close-up of the larvae, which always move to cluster together. **c)** *Anisakis pegreffii* coils up when dormant. **d)** An *Anisakis pegreffii* L₃, "awake".

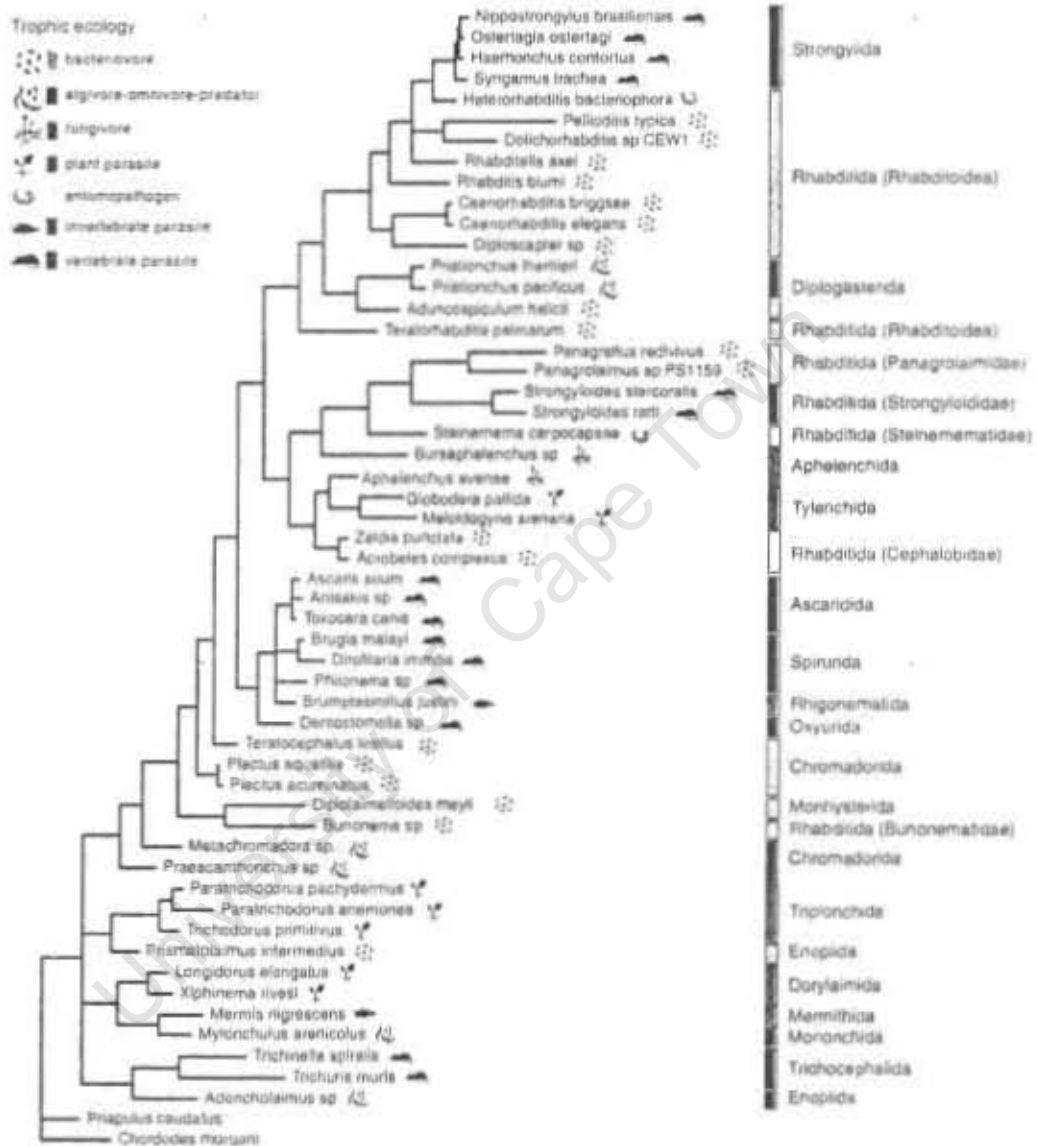


Figure 1.3. A phylogenetic tree of nematode species. Phylogenetic analysis was performed on small subunit ribosomal DNA sequences from a wide range of nematodes. The results indicate that *Anisakis* spp are closely genetically related to *Ascaris suum* and *Toxocara canis*. From: Blaxter (1998)²⁸.

L₂ larvae that are ingested by tiny crustaceans such as krill (e.g. *Euphasia*, *Tysanoessa*), the first intermediate hosts^{16, 27, 37}. The crustaceans in turn are eaten by second intermediate hosts, which are fish, larger crustaceans or cephalopods. Inside these hosts, the larvae moult into third-stage larvae and become encapsulated on the surfaces of organs or muscles. Larger fish may become infected by eating smaller fish, leading to an accumulation of larvae that increases with the age of the fish. All kinds of L₃-infected seafood can cause anisakiasis when ingested by humans. Humans are an “accidental host” in which the larvae cannot complete their life-cycle; other accidental hosts include bears, otters and cats³⁸⁻⁴⁰. In the natural cycle, the third-stage larvae in fish are ingested by cetaceans and moult into fourth-stage larvae and then adults. They cluster inside the stomachs of the cetaceans, where the female adult worms are fertilized and lay eggs, completing the cycle. Occasionally, anisakids moult into fourth-stage larvae in humans, but do not progress into adults. *Pseudoterranova* spp. are more likely to moult into fourth-stage larvae than *Anisakis* spp. In a rare case, an adult male worm of *Pseudoterranova* was found in a patient⁴¹. This is regarded as an exception⁴².

1.3 Disease caused by *Anisakis* species

Live *Anisakis* L₃ are capable of infecting humans to cause a disease known as anisakiasis. This disease, which will be discussed in more detail in Chapter Two, normally presents as mild to severe abdominal pain, nausea, vomiting and/or diarrhoea, and is in some cases accompanied by allergic reactions such as urticaria (hives), angioedema, bronchospasm and even severe anaphylaxis⁴³⁻⁴⁵. The disease therefore lies at an interesting junction between parasitic infection and allergic response. Many facets of the immune response to helminths and allergens are similar, but the two diseases do not usually coalesce. In fact, the current thought is that helminth infection may suppress symptoms of allergy.

Apart from allergic symptoms occurring during acute infection with *Anisakis*, there are also several case reports of allergy to *Anisakis* proteins occurring in the occupational or domestic setting, with symptoms such as asthma, rhinitis, conjunctivitis and dermatitis⁴⁶⁻⁵⁰. There are also some cases where consumption of cooked or canned fish appears to

have led to *Anisakis*-specific allergic reactions⁵¹. Skin prick testing with deep-frozen and heat-treated or boiled extracts has demonstrated the resistance of *Anisakis* allergens to cooking, and many *Anisakis* allergens are also resistant to degradation by the digestive enzyme pepsin^{44, 52}. This suggests that reactions might occur after exposure to *Anisakis* antigens alone (vs live larvae)¹⁶. However, many authors consider reactions to *Anisakis* a pseudo-allergy that occurs due to inflammatory responses generated during acute infection and requires a live parasite⁵³. Studying the immune and allergic responses to *Anisakis* may help resolve this issue.

1.4 Prevention of anisakiasis

The best means of avoiding infection with *Anisakis* or related nematodes is to ensure that all fish to be consumed raw or partially cooked is deep-frozen (-20°C) for at least 24 hours⁵⁴, and that cooked fish reaches a temperature of at least 60°C throughout for 10 minutes or over¹⁶. The number of cases of anisakiasis in the Netherlands has dropped to almost zero since these measures were included in the legislation⁵⁵. Other countries have taken similar measures. Current European Community regulations require visual examination of fish with removal of heavily parasitized specimens from the market, and extraction of visible larvae in less heavily parasitized specimens, as well as freezing of fish to be consumed raw or undercooked to -20°C for 24 hours throughout the entire product¹⁶. Food and Drugs Administration (FDA) regulations in the United States require that all fish and shellfish that will not be cooked or processed at temperatures above 60°C are blast-frozen to -35°C or below for 15 hours or frozen at -23°C or below for 7 days⁵⁶. According to the South African Bureau of Standards, international food safety laws are applied in South Africa⁵⁷. While such measures may make commercially available frozen fish safe, fresh fish can be bought or caught by consumers, and such stringent procedures may not be followed in the domestic environment. Fresh fish that was presumed to be well-cooked is often the source of infection in reported cases¹⁶. Furthermore, freezing of fish alters the flavour of sushi and other fish delicacies, so the practice may be intentionally avoided⁴². This means that *Anisakis* infections are likely to continue to occur in the future despite legislation.

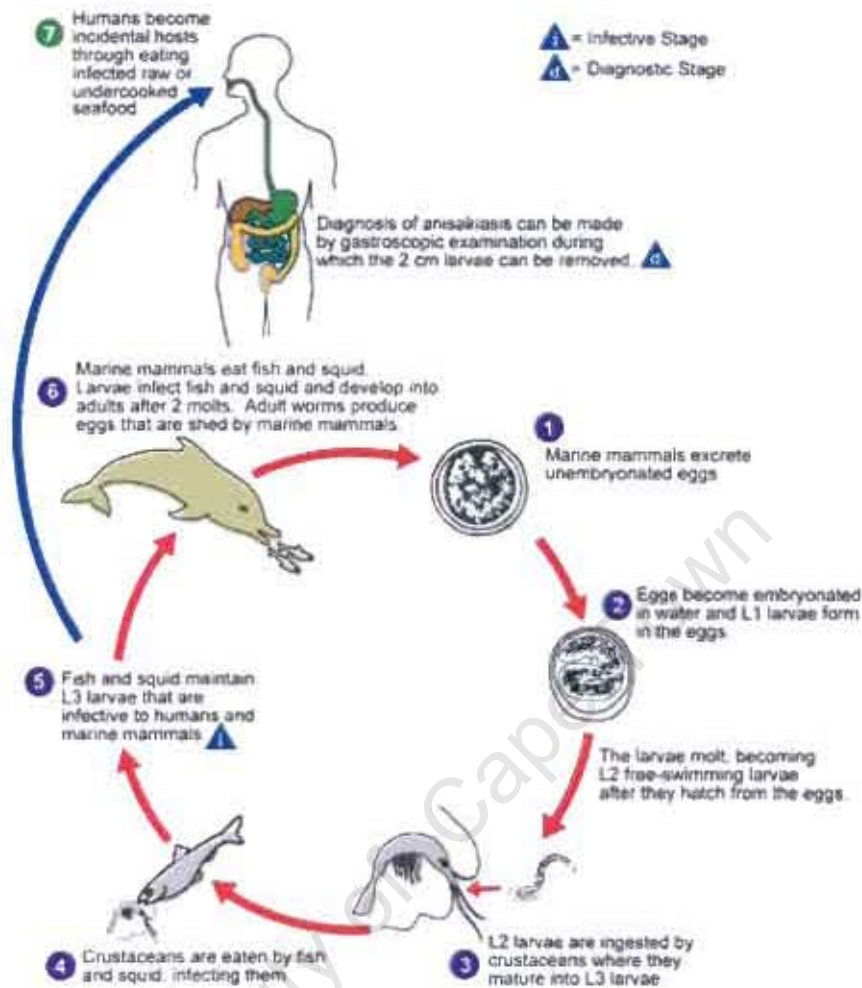


Figure 1.4. The lifecycle of *Anisakis* and other anisakids. *Anisakis* eggs enter the water in the faeces of cetaceans. Inside the eggs, the larvae develop into L₂ stage larvae before hatching. L₂ are ingested by tiny crustaceans such as krill. In turn, the krill are eaten by fish, cephalopods and large crustaceans, the second intermediate hosts in which the larvae develop into L₃ stage. Finally, L₃ larvae are ingested by the primary host, a cetacean. They moult into adults, and begin to reproduce. L₃ can be accidentally ingested by humans or other land mammals, but are not adapted to these hosts and cannot complete their life cycle. Picture from the website of the Centre for Disease Control (CDC) ³⁷.

1.5 The immune response to intestinal nematodes

1.5.1 Innate and adaptive immune responses

Immune responses to pathogens are characterized by innate immune responses, which are the first line of defence and are rapidly activated, and adaptive immune responses, which develop more slowly and provide a tailored response directed against specific antigens⁵⁸. The innate immune system is composed of NK cells, dendritic cells (DCs), macrophages, neutrophils, eosinophils, mast cells, basophils, platelets and the complement system^{58,59}. Upon a primary exposure to a helminth various degrees of inflammatory responses are triggered, characterized by the recruitment of inflammatory effector cells such as neutrophils and eosinophils and changes in blood vessel permeability and blood flow^{60,61}. These changes can be initiated by the activation of the alternative complement pathway and the non-specific degranulation of mast cells. Basal levels of eosinophils are always present and the presence of helminths can cause them to rapidly infiltrate to the site of infection^{62,63}.

Non-specific inflammation is minimal in most natural host-parasite systems, but is often enhanced in unnatural or non-permissive hosts⁶⁰. *Anisakis* species and *Ancylostoma caninum*, a canine hookworm, are examples of unnatural parasites of humans that can elicit significant eosinophilic enteritis during a primary infection^{64,65}. This non-specific inflammatory response may be the principal mechanism of limiting primary infections in unnatural host-parasite systems⁶⁰. Natural parasites have evolved evasive mechanisms and immunosuppressive molecules, and enhanced effector responses mediated by the adaptive immune system are required for their expulsion⁶⁶⁻⁶⁸.

The adaptive immune system is composed of lymphocytes, of which the two major classes are T cells and B cells⁶⁹. B cells are responsible for producing antibody, while T cells can destroy diseased cells (cytotoxic T cells), prevent excessive immune reactions (regulatory T cells) and orchestrate immune responses by the secretion of an array of immunoregulatory molecules called cytokines (helper T cells). Antigen-presenting cells (APCs) such as dendritic cells and macrophages continually process and present intracellular and extracellular proteins for inspection by lymphocytes⁷⁰. If a lymphocyte

recognizes a foreign antigen it is activated to proliferate and differentiate. Prior to the release of lymphocytes into the circulation, regulatory mechanisms ensure that lymphocytes which recognize "self" antigens are eliminated^{71,72}.

In recent years a number of pattern-recognition receptors (PRRs) have been identified on APCs, specific to components that may be found in certain classes of pathogen such as bacterial DNA and components of bacterial and fungal cell walls⁷³⁻⁷⁵. When a PRR binds to such a component, pathways are triggered in the APC that cause changes in gene expression, influencing the function and behaviour of the APC^{70, 76}. This includes changes in the expression of chemokine receptors, cytokines and costimulatory molecules. The immediate cytokine environment during antigen presentation and the differential expression of costimulatory molecules on APCs influence the differentiation of naïve T cells into particular T cell subsets^{77, 78}. Therefore different classes of pathogens can specifically elicit certain subsets of T helper cells and consequently induce the effector responses associated with that subset. As helminth infections are consistently associated with adaptive Th2 type responses, it is possible that some feature(s) common to all nematodes is recognized by the host⁷⁹.

1.5.2. The role of CD4⁺ T helper cells in resistance to intestinal nematodes

Human epidemiological studies and experimental mouse models both support the idea that repeated infection by helminths can lead to acquired protective immunity in the host⁷⁹⁻⁸¹. CD4⁺ T helper (Th) cells play an important role in mediating the adaptive immune response by the secretion of a wide variety of cytokines, and are known to be essential for resistance to nematodes⁶⁸. A naïve T cell can differentiate into one of several T cell subsets, defined by their cytokine-expression profiles^{77, 82, 83}. Current models describe four major subsets: Th1, Th2, Th17 and T regulatory(reg) cells^{82, 83} (Fig. 1.5). Th1 cells are associated with the activation of cytotoxic responses and resistance to intracellular parasites, while Th2 cells are associated with allergies and resistance to helminths^{68, 77}. Th17 cells are proinflammatory and associated with autoimmune diseases and resistance to extracellular pathogens, and Treg cells are anti-inflammatory and are associated with immunosuppression^{82, 83}. IL-12 promotes differentiation towards Th1 cells, while IL-4

promotes the development of Th2 type cells^{77, 84, 85}. Th2 and Th1 responses are mutually antagonistic and recent evidence suggests that there may be a similar relationship between Treg (anti-inflammatory) and Th17 (pro-inflammatory) cells^{77, 82, 83}. Transforming growth factor(TGF)- β in the absence of IL-6 promotes the development of Treg cells, while the combination of TGF- β , IL-6 and IL-23 promotes Th17 cells^{82, 83}.

In mouse models, resistance to helminths is associated with the activation of Th2 cells and the production of the Th2 type cytokines IL-4, IL-5, IL-9, IL-10 and IL-13, while the activation of Th1 cells and the presence of IFN- γ are associated with prolonged or chronic infection^{67, 68, 80, 86-89}. Similarly, in humans helminths induce Th2 type responses, which are associated with reduced worm burdens^{12, 90}. In addition, helminth infections are often associated with Treg cells, regulatory B cells and the production of IL-10⁹¹. The associated immunosuppression may protect parasites against inflammatory host defences and also benefit humans to an extent by limiting harmful inflammation^{12, 66, 91-96}. While IL-10 is often associated with Th2 responses in helminth infections^{91, 97}, there is some evidence that IL-10 associated Th2 responses are modified, with production of IgG rather than IgE, compromised specific T cell proliferation, decreased levels of IL-5 and eosinophils, and inhibition of mast cell degranulation and cytokine production^{95, 98, 99}.

1.5.3. Effector mechanisms in immunity to intestinal nematodes

Much of our knowledge about immunity to intestinal nematodes is gleaned from laboratory models of intestinal nematode infection⁶⁸. In mice, helminth infections are associated with eosinophilia, mastocytosis, goblet hyperplasia, high total (polyclonal) IgE production, helminth-specific IgE and IgG1 production and the accumulation of alternatively activated macrophages^{60, 79, 91, 100, 101}. Similar features are evident in humans and livestock^{102, 103}. Experimental models indicate that the effector mechanisms required for expulsion or destruction of nematodes vary between species and between larval and

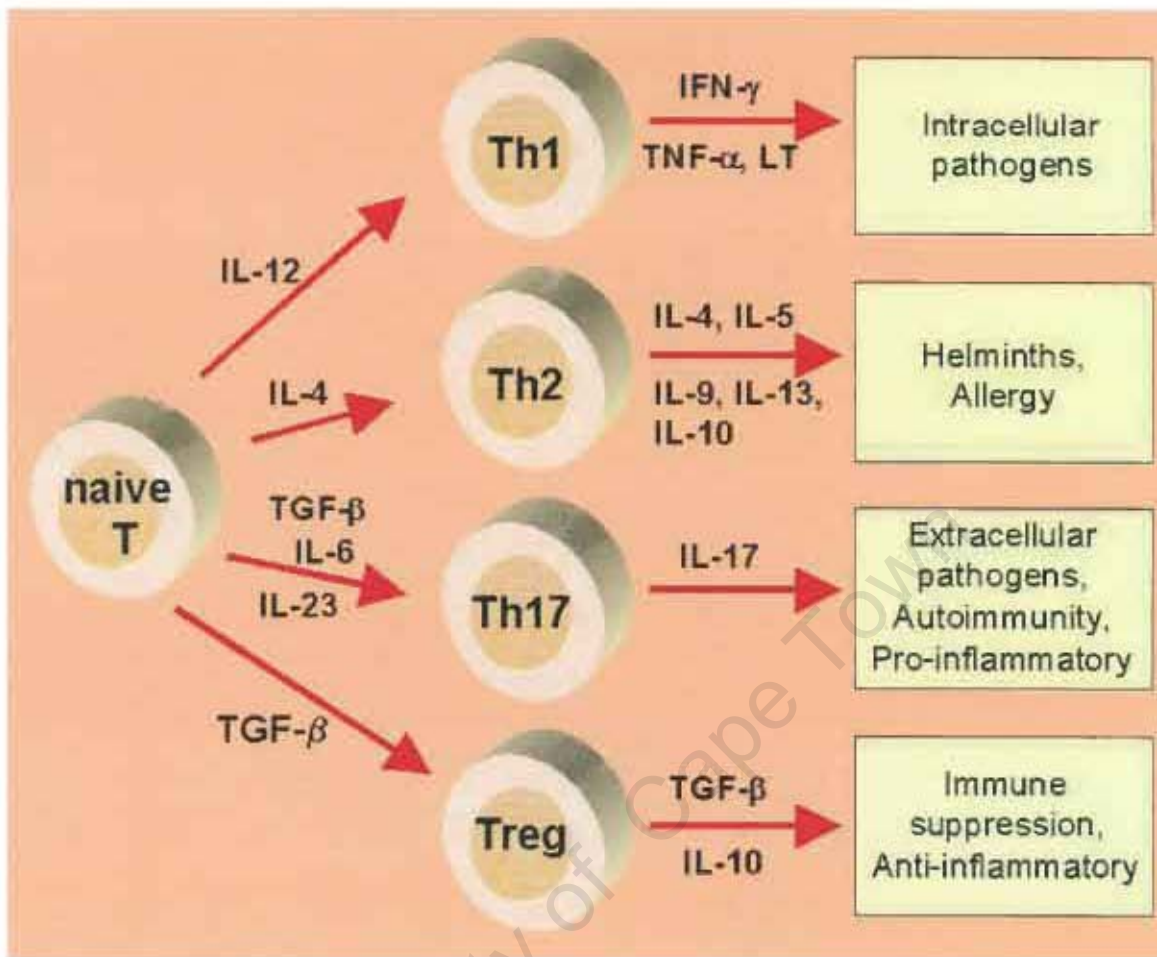


Figure 1.5. T helper cell subsets. Naïve T cells can differentiate into distinct T cell subsets as a result of different environmental conditions during antigen presentation. IL-12 favours differentiation into Th1 cells, which are important in defence against intracellular pathogens, while IL-4 induces differentiation into Th2 cells, associated with helminth immunity and allergy. The combined presence of TGF- β and IL-6 induces the pro-inflammatory Th17 subset, while TGF- β in the absence of IL-6 promotes the development of Tregs, important mediators of immune suppression. IL-23 promotes Th17 differentiation. Based on a figure by Tato and O'Shea⁸².

worm forms of the parasite^{60, 68, 79, 104}. For example, eosinophils appear to play a role in the damaging or killing of infective L3 larvae, but not in the expulsion of mature worms, except perhaps in unnatural or non-permissive hosts⁶⁰. Eosinophils release potent mediators such as eosinophil peroxidase and major basic protein that can damage and kill helminth larvae¹⁰⁵. IL-5 released by Th2 cells stimulates eosinophil production and differentiation and increases the killing capacity of eosinophils¹⁰⁶⁻¹⁰⁸. Specific antibody is thought to enhance eosinophil-mediated killing of larvae⁶⁰.

In contrast, mast cells and goblet cells often play a role in the expulsion of intestinal dwelling adult worms^{60, 68}. Mastocytosis is controlled by Th2 cytokines, including IL-3, IL-4, IL-9, IL-10 and the growth factor stem cell factor (SCF)^{68, 109}. Mast cells release multiple mediators, including proteases, cytokines, histamine and leukotrienes, and disrupt gut epithelial barrier function^{110, 111}. In particular, it has been shown that mouse mast cell protease-1 (MMCP-1) increases intestinal permeability, presumably by degrading a component of the tight junction between gut epithelial cells, leading to an increase in intraluminal fluid that may help flush out parasites¹¹¹. Both IL-4 and IL-13 are thought to act as effector molecules in increasing intestinal permeability and contractility^{80, 109, 112, 113}. Experimental data has shown that IL-13 plays an important and sometimes critical role in expulsion, although the precise mechanisms have yet to be fully elucidated⁷⁹. It has been shown that goblet cell hyperplasia is dependent on IL-13¹¹⁴⁻¹¹⁶. Goblet cells secrete mucins that help trap and expel intestinal nematodes from the gut⁶⁸. Enhancement of epithelial cell turnover by IL-13 also appears to play a role in helminth expulsion¹¹⁷.

A fairly new area of research explores the role of alternatively activated macrophages as effector cells in helminth infections^{100, 118, 119}. In contrast to classically activated macrophages, which are activated by IFN- γ and TNF to produce microbicidal NO, alternatively activated macrophages are activated by IL-4 and IL-13 (and to a lesser degree by IL-10) to express the enzyme arginase-1^{100, 120} (Fig. 1.6). Arginase-1 and NO synthase (NOS-2) compete for the same substrate, L-arginine¹²¹. NOS-2 converts L-arginine into NO and citrulline while arginase-1 converts it into urea and L-ornithine,

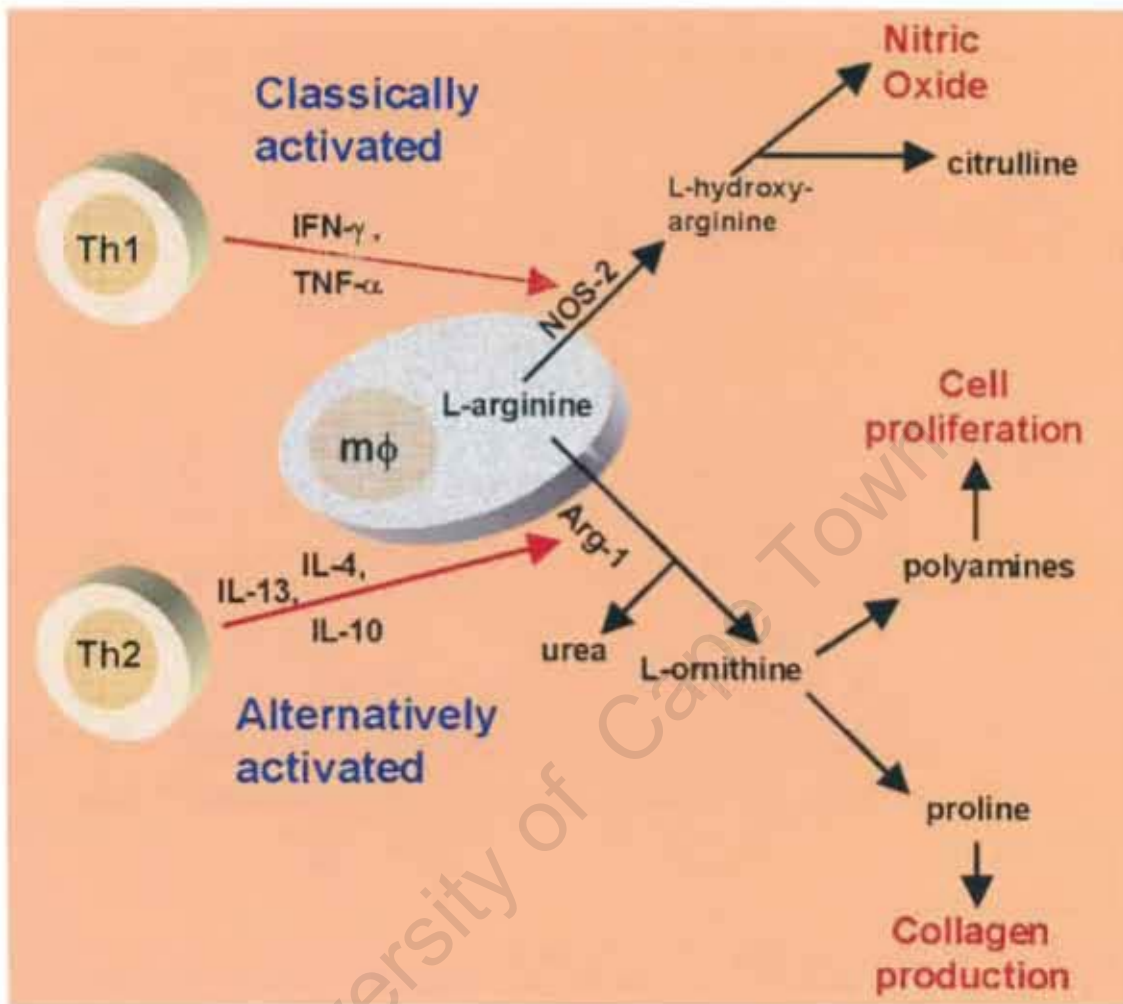


Figure 1.6. Alternative macrophage activation. The enzymes nitric oxide synthase (NOS-2) and arginase-1 (Arg-1) compete for the substrate L-arginine. In “classically activated” macrophages, Th1 type cytokines activate nitric oxide synthase (NOS-2), which catalyzes the production of antimicrobial nitric oxide (NO). In “alternatively activated” macrophages, Th2 cytokines activate arginase-1 (Arg-1), which catalyzes the production of urea and L-ornithine. L-ornithine acts as a substrate for the generation of polyamines and prolines, metabolites in cell proliferation and collagen production, respectively. Adapted from Wynn TA (2003) ¹²².

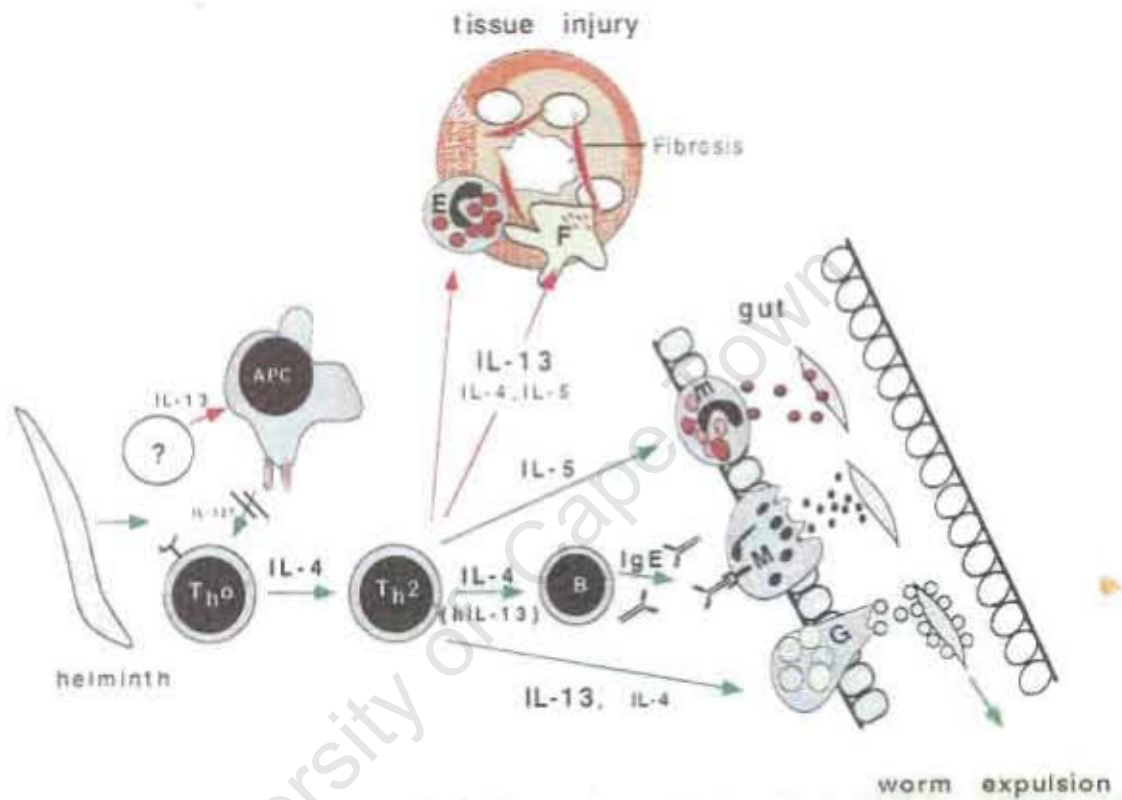


Figure 1.7. Effector mechanisms in helminth expulsion. The Th2 cytokines IL-4, IL-5, IL-9 and IL-13 enhance the generation and activation of effector cells such as eosinophils, mast cells and goblet cells. Eosinophils release toxic products involved in killing the larval stages of helminths, while mast cells and goblet cells assist in the expulsion of intestinal-living worms by increasing intestinal permeability and secreting mucus, respectively. Th0 = naïve T cell, Th2 = T helper 2 cell, B = B cell, E = eosinophil, M = mast cell, G = goblet cell. From Brombacher F (2000)¹²³

a metabolite required for the production of proline and polyamines. Proline is required for collagen deposition, while polyamines play an essential role in cell proliferation¹²⁰. These metabolites are implicated in fibrotic diseases but their effects in helminth infection are still being explored^{100, 118, 120}. However, it has been shown that arginase-1 activity mediates host protective responses to the intestinal nematode *Heligossomoides polygyrus*¹¹⁸. Other metabolic products produced as a result of alternative macrophage activation and/or arginase-1 induction may also play a role in immunity to helminths¹²⁴.

In summary, Th2 cytokines orchestrate a number of intestinal changes that render the gut lumen environment intolerable for the establishment or survival of nematodes¹² (Fig. 1.7). As some helminths have larval stages that migrate through the lungs, similar effector responses are initiated in the airways¹³. Interestingly, the same effector mechanisms involved in resistance to helminths are often involved in the pathology of allergy⁹¹. It is speculated that Th2 responses evolved to manage helminth infections, but in the absence of helminth-induced regulatory mechanisms can result in allergic responses to harmless proteins⁹⁵.

1.6 The allergic immune response

Allergy is also termed type 1 hypersensitivity (immediate type hypersensitivity) and is one of four classes of hypersensitivity reactions that involve tissue damage due to immunological mechanisms¹²⁵. It is mediated by specific IgE antibodies produced after exposure to harmless proteins present in the surroundings. Certain proteins (allergens) are more likely to elicit an IgE response, and certain people (atopics) have a genetic tendency to produce specific IgE to these proteins and subsequently develop allergic reactions¹²⁶.

Allergy can be divided into a sensitization and an effector stage¹²⁷. When an allergen comes into contact with the immune system, it is processed by APCs and presented to naïve T cells (Fig. 1.8). In an IL-4-dominated cytokine environment, naïve T cells differentiate into Th2 cells which produce IL-4, IL-5, IL-9 and IL-13, cytokines which play a central role in allergic disease^{84, 128, 129}. Allergic disease is associated with activation of Th2 cells, IgE production, eosinophilia, mast cell degranulation, vascular

and intestinal permeability, goblet cell hyperplasia, mucus hypersecretion and fibrosis¹²⁷. Genetic variants in IL-4, IL-13 and the IL-4 receptor are associated with atopy and asthma^{130, 131}.

IL-4 and IL-13 induce IgE isotype switching in human B cells, while IL-4 induces IgE switching in mice^{123, 132, 133}. An individual with specific IgE against an allergen is said to be sensitized to that allergen. Specific IgE binds to the FcεR1 on the surface of mast cells and basophils¹³⁴. A second exposure to the same allergen cross-links this bound IgE, causing the mast cells or basophils to degranulate, releasing potent inflammatory or vasoactive mediators such as histamine, leukotrienes, cytokines, serotonin, PAF, and various proteases^{135, 136}. This is known as the effector stage. The mediators released from mast cells are primarily responsible for the acute symptoms of allergy, which can include itching, urticaria, angioedema, rhinitis, conjunctivitis, asthma, dermatitis, gastrointestinal disturbances, and anaphylactic shock^{136, 137}. In addition, released mediators stimulate inflammatory cell proliferation, recruitment and accumulation¹³⁷⁻¹³⁹.

Asthma is perhaps the most well studied of the allergic diseases, and mouse models illustrate how Th2 effector mechanisms contribute to its allergic pathology (Fig. 1.9) The symptoms of acute asthma are caused by excessive mucus production in the lungs, acute inflammation and broncho-constriction due to the release of mediators by mast cells^{115, 127, 137}. These mechanisms narrow the airways and limit airflow, causing breathing problems¹²⁷. Chronic asthma is caused by chronic inflammation and tissue damage that leads to airway remodeling through collagen deposition, smooth muscle hypertrophy and epithelial cell shedding^{127, 140}. These changes can severely decrease lung function.

It is thought that IL-13 is the central mediator of allergic asthma^{115, 116, 127, 141}. Mice which overexpress IL-13 show elevated specific IgE, mast cell degranulation and histamine release, and also show allergen-induced pathology in the lungs including goblet cell hyperplasia and mucus hypersecretion, fibrosis, eosinophilia and raised levels of Th2 cytokines¹⁴². The release of toxic products by eosinophils and other inflammatory cells play an important role in ongoing inflammation⁶⁰. CD4⁺ T cells play a crucial role in

remodeling of the airway wall, possibly by producing IL-13, which induces fibrosis^{120, 140, 143}. There is evidence that alternatively activated macrophages play an important role in fibrosis and tissue remodeling by producing proline, a precursor of collagen¹²⁰. Similar effector mechanisms may play a role in other allergic diseases such as rhinitis, eczema and food allergies, and this is a subject of ongoing research.

1.7 Helminths and allergy: The hygiene hypothesis

The dramatic increase in the prevalence of allergy over the last few decades has occurred predominantly in the developed world, suggesting that environmental factors may be responsible¹⁴⁴. In 1989, Strachan proposed the “hygiene hypothesis” based on epidemiological data that associated increased sanitation and reduced childhood infections with an increased risk of allergy¹⁴⁵. This hypothesis proposes that early infections or exposure to microbial products such as endotoxin reduce the risk of allergy by skewing immune responses towards Th1 and away from Th2 responses¹⁴⁶.

However, more recently it has been shown that the prevalence of allergy is low in populations with high prevalences of Th2-inducing helminth infections⁹⁹. In addition, Th1-mediated autoimmune diseases such as Crohn’s disease, inflammatory bowel syndrome, multiple sclerosis and type 1 diabetes are on the rise in the same sanitized, urbanized populations that are experiencing an increased prevalence of allergy^{147, 148}. This has led to a variation of the hygiene hypothesis in which infections by helminths (and possibly other microbes) are thought to induce suppressive regulatory responses that modulate both excessive Th2 and Th1 responses^{94, 149, 150}. These suppressive responses are thought to involve the “anti-inflammatory network”, consisting of T regulatory cells and the cytokine mediators IL-10 and TGF- β ^{94, 95}.

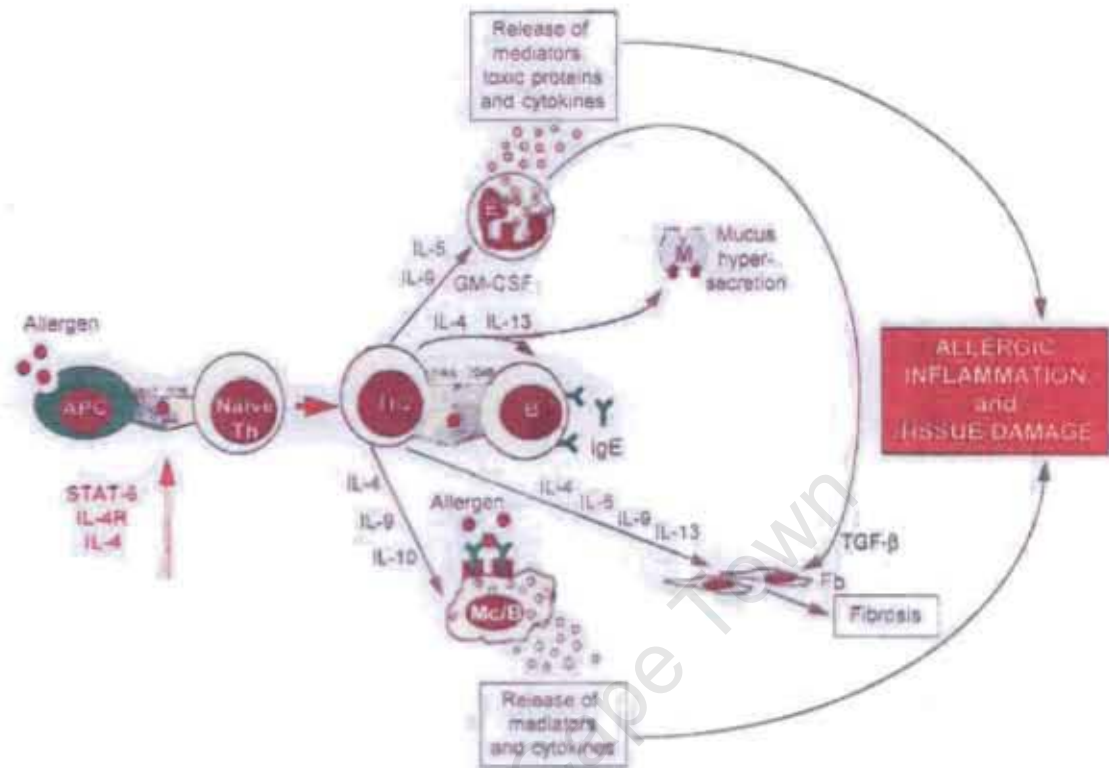


Figure 1.8. Basic mechanisms of allergy. Antigen presenting cells process and present incoming allergens to naïve T cells. In the presence of IL-4, naïve T cells differentiate into Th2 cells which produce a wide range of pro-allergic cytokines. These include IL-4, IL-5, IL-9 and IL-13, which enhance the generation and activation of effector cells such as mast cells, eosinophils and goblet cells. IL-4 and IL-13 stimulate B-cell production of IgE, which binds to FcεRI receptors on mast cells and basophils. Upon a second exposure to the allergen, bound IgE molecules are cross-linked, causing the degranulation of mast cells and basophils and the release of inflammatory mediators such as histamine, leukotrienes, cytokines and prostaglandins. Furthermore, IL-4 and IL-13 stimulate the growth and chemotaxis of fibroblasts and the synthesis of extracellular matrix proteins, while IL-5, IL-9 and TGF-β stimulate subepithelial fibrosis. Together this can lead to tissue remodelling. APC = antigen presenting cell, Th = T helper cell, Th2 = T helper cell type 2, B = B cell, E = eosinophil, Mc/B = mast cell/basophil, Fb = fibroblast, M = mucus. Figure adapted from Romagnani S (2000)¹²⁹.

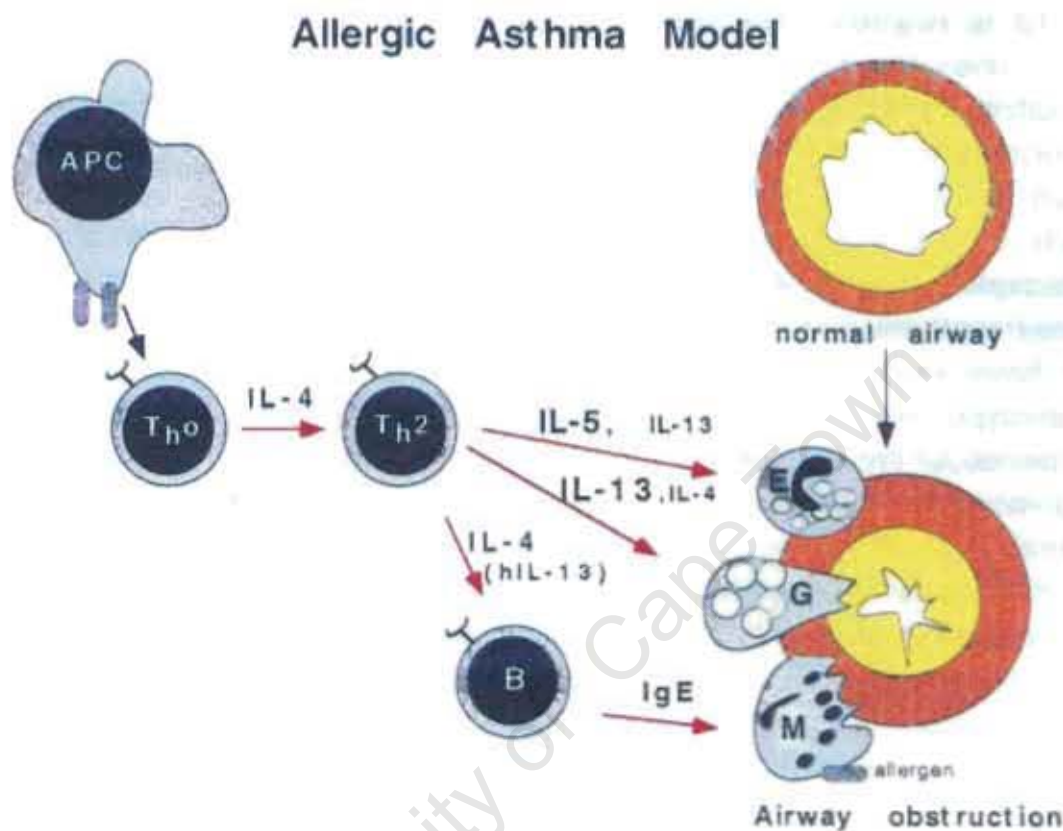


Figure 1.9. Th2 effector mechanisms in allergic asthma. The same Th2 effector mechanisms that assist in helminth defence are detrimental during allergic asthma. Mucus hypersecretion by goblet cells and inflammatory mediator release by mast cells contribute to airway obstruction. Eosinophils release toxic and inflammatory mediators that cause tissue damage in the lung, leading to airway remodeling. APC = antigen presenting cell, Th0 = naïve T helper cell, Th2 = T helper 2 cell, B = B cell, E = eosinophil, G = goblet cell, M = mast cell. From Brombacher F (2000) ¹²³.

Various cross-sectional epidemiological studies have shown an inverse correlation between helminth infection and allergic sensitization¹⁵¹⁻¹⁵⁵, and other studies have demonstrated an increase in atopy (measured as positive skin prick test results to one or more aeroallergens) following anti-helminthic treatments^{152, 156, 157}. However, there are also many studies that have shown no correlation or even a positive correlation between helminth infection and atopy or allergic disease¹⁵⁸⁻¹⁶², and no effect of antihelminthic treatment on atopy¹⁶³. It has also been suggested that an inverse association between atopy and helminth infections could be due to reverse causation: that is, an atopic state is protective against helminth infection and people with chronic helminth infection are more likely to be non-atopic^{95, 164}.

Despite these inconsistencies, it is clear that helminths can induce strong immune suppressive responses^{9, 66, 91, 165, 166}, and many experimental murine models have demonstrated protective effects of helminths against allergy, shown to be mediated by T regulatory cells and/or IL-10^{13, 91, 167-171}. To explain the disparities between the various field studies, it has been suggested that the intensity and duration of infection, age at infection and species of helminth may influence the effects of the helminth on the immune system^{172, 173}. Chronic infection and/or a high parasite burden is thought to induce regulatory immune responses, resulting in a suppression of allergic disease, while low-level and/or transient infections may exacerbate allergic disease by stimulating Th2 responses¹⁷².

A recent review and meta-analysis of 33 helminth-allergy studies from 1962 onwards (including n = 25 753 patients) found that overall, helminth infection was not protective against asthma/wheeze; however, hookworm was significantly associated with protection against asthma that correlated to the intensity of infection¹⁶⁰. In contrast, *Ascaris lumbricoides* was significantly associated with an increased risk of asthma. This illustrates that different species of helminth may have different effects on asthma and other allergic diseases, in an intensity-dependent manner. In experimental mouse studies, the suppressive effects of different helminth species on allergies do not share a common mechanism, with IL-10 being the protective mediator in some cases and not in others⁹¹.

^{168, 169, 171}. Much as there are differences in the mechanisms of worm expulsion between species ⁶⁸, so do there appear to be differences in the regulatory mechanisms stimulated by different helminth species. The outcome of any parasite-allergy interaction appears to depend upon the balance between parasite induction of suppressive regulatory (IL-10) responses and detrimental (IL-4) allergic responses ¹⁷¹.

Many epidemiological studies designed to explore the association between atopy and helminth infections do not control for confounders, such as the prevalence of other infectious diseases in helminth-infected populations ¹⁷⁴. Th1-polarizing infections such as tuberculosis and faecal-oral infections have also been associated with a reduced incidence of atopy/allergy in humans and mice ¹⁷⁵⁻¹⁷⁸. Experimental data show that microbes and their products can also induce regulatory/suppressive responses ¹⁷⁹. It is therefore possible that infections with helminths, bacteria and viruses combine to stimulate the regulatory responses necessary for the prevention of allergic disease ^{150, 174, 179}. Experimental data will help unravel the mechanisms of this regulation, and may even reveal immunomodulatory molecules from helminths and other infectious agents that could be used in therapy against allergies and autoimmune diseases ¹⁷⁴. It has already been shown that deliberate *T. suis* infection is safe and effective in alleviating inflammatory bowel disease ¹⁸⁰, and trials are planned involving the administration of hookworm to patients with asthma or other allergies ¹⁸¹.

1.8 A common receptor for IL-4 and IL-13: the IL-4 receptor alpha

A range of experimental models and epidemiological studies have established the importance of IL-4 and IL-13 in helminth infection and in allergy. The genes for IL-4 and IL-13 are closely linked on both human and mouse chromosomes ¹⁸². IL-4 and IL-13 have low amino acid homology (about 30%) but very similar tertiary structures ^{182, 183}, and share a receptor subunit, the IL-4 receptor alpha (IL-4R α) ¹²³. The IL-4R α can pair with the common γ chain subunit, a component of many cytokine receptors, or with IL-13R α 1 or IL-13R α 2 (Fig. 1.10)¹⁸⁴. The combination of IL-4R α and the common γ chain is known as the IL-4 receptor type 1, and is utilized by IL-4 only. The IL-4R α subunit paired with the IL-13R α 1 forms a complex known as IL-4 receptor type 2 or IL-13

receptor, through which both IL-4 and IL-13 can signal. The IL-13R α 1 on its own binds IL-13 with only weak affinity. However, when IL-13R α 1 combines with IL-4R α , the resulting complex has a high affinity for IL-13. Experimental data suggests that both IL-4 and IL-13 induced signal transduction occurs through the IL-4R α . However recent data suggests that IL-13 may also signal through the IL-13R α 2¹⁸⁵.

Upon binding of IL-4 to the IL-4R α or binding of IL-13 to the IL-4R α /IL-13R α 1 complex, a conformational change occurs in the IL-4R α that brings together receptor-associated Janus kinases (JAKs). JAK-dependent tyrosine phosphorylation of the IL-4R α occurs, and the IL-4R α subsequently phosphorylates the transcription factor known as signal transducer and activation of transcription (STAT) 6, enabling it to translocate to the nucleus where it activates the transcription of many Th2-associated genes^{184, 186}. Other signalling intermediates such as insulin receptor substrate (IRS)-1/2 can also be phosphorylated by the IL-4R α ¹⁸⁴. Due to this common signalling pathway, IL-4 and IL-13 share many biological functions, including induction of B-cell isotype switching to IgE, activation of alternative macrophages and inhibition of inflammatory macrophage products, upregulation of major histocompatibility complex (MHC) class II and promotion of Th2 type responses^{123, 183, 187}.

Both IL-4 and IL-13 are predominantly produced by activated CD4⁺ Th2 cells, mast cells and basophils, although these are not their only cellular sources¹²³. Although there is overlap between IL-4 and IL-13 functioning, the two cytokines also have distinct functions. This is due in part to the differential expression of the various receptor components on different cell types. For example, T cells do not express IL-13R α 1, and can therefore not respond to IL-13. As a result, IL-4 can directly induce Th2 cell differentiation, but IL-13 cannot, although it can indirectly promote Th2 type responses^{182, 188-190}. Differences in IL-4 and IL-13 functioning may also occur due to the complexity of receptor usage, which can result in differences in downstream signalling¹⁸⁹. For example, IL-4 signals through both the IL-4R type 1 and the IL-4R type 2, while the IL-13 can bind to both IL-13R α 1 and IL-13R α 2^{185, 191}. Furthermore, soluble forms of

the IL-4R α and the IL-13R α also occur and are thought to regulate IL-4 and IL-13 responses^{123, 192}.

Due to their association with allergy, IL-4, IL-13 and their receptors are potential therapeutic targets. Several clinical trials using neutralizing antibodies have already taken place while others are in progress^{127, 193}. While anti-IL-4 monoclonal antibodies were not effective at preventing asthma, soluble IL-4R α antagonists did show clinical benefits^{127, 194}. Recent experimental data indicates that IL-13 plays a distinct and central role in effector mechanisms in allergic asthma^{115, 116, 141, 195}. Apart from being a central mediator of goblet cell hyperplasia and mucus hypersecretion in the lung, it also appears to play an important role in fibrosis associated with airway remodeling^{187, 195, 196}. Phase I trials using anti-IL-13 against asthma as well as the development of anti-IL-13 receptor antibody are in progress^{193, 197}. However, some mouse studies indicate that blocking of IL-4 and IL-13 in combination may be more effective^{127, 198}. There are indications that IL-13 may have effects in the absence of the IL-4R α ¹⁹⁹. In addition, IL-4R α $-/-$ mice develop IgE and allergic responses after prolonged sensitization^{200, 201}. As studies indicate that different allergic diseases and routes of sensitization involve variable roles for IL-4 and IL-13, therapeutic approaches should be accompanied by a full understanding of the mechanisms involved.

1.9 Animal models of allergy

Allergy is a complex and multifactorial disease that requires interaction between a number of different cell-types and mediators. As such it is difficult to unravel mechanisms of allergy *in vitro*. For example, while IL-4R α was shown to play a profound role on the activity of smooth muscle cells *in vitro*, the *in vivo* loss of IL-4R α on smooth muscles cells did not influence airway hyperreactivity or airway inflammation²⁰². This makes it difficult to assess the significance of *in vitro* results in relation to human disease. Similarly, data acquired in models using animals such as mice cannot be extrapolated directly to humans because of species-specific differences. However, with this caveat in mind, animal models provide a closer simulation of the human system than *in vitro* studies.

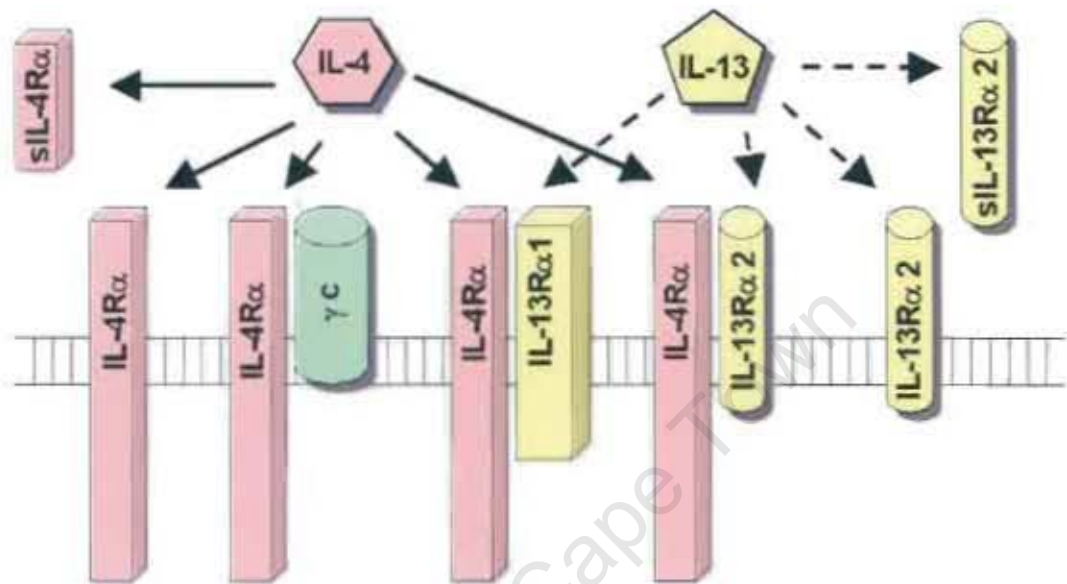


Figure 1.10. IL-4 and IL-13 share a complex receptor system. IL-4 binds to the IL-4R α chain, which can combine with the common gamma (γ) chain receptor, IL-13R α 1 or IL-13R α 2. IL-13 binds to the IL-13R α 1 and IL-13R α 2, both of which can complex with the IL-4R α chain. Signal transduction for both cytokines occurs through the IL-4R α . The role of IL-13R α 2 is incompletely understood. Soluble forms of the IL-4R α (sIL-4R α) and IL-13R α 2 (sIL-13R α 2) also exist and may modulate IL-4/IL-13 responses. Figure adapted from Brombacher (Brombacher 2000)¹²³.

To date, numerous experimental models of allergic disease have been established in mice, including models of asthma, dermatitis, food allergy, anaphylaxis and rhinoconjunctivitis. Many scientific groups have progressed from using wild-type mice to using transgenic or knock-out mice which over-express or lack specific genes, in order to determine the function and importance of specific proteins with the ultimate aim of designing new therapies²⁰³. In this project, previously generated mice deficient in IL-4²⁰⁴, IL-13¹¹⁴, IL-4/IL-13¹⁸⁹ or the IL-4 receptor alpha (IL-4R α)²⁰⁵ have been used in order to elucidate mechanisms of *Anisakis*-associated disease. In certain models, cell-specific requirements for IL-4R α have been investigated using recently generated macrophage/neutrophil or T-cell specific IL-4R α deficient mice.

1.10 Cell specific knockout mice

In recent years a genetic technique has been developed that allows cell-specific gene deletion. This technique employs the DNA sequences and enzymes that bacteriophage P1 uses to excise itself from a host cell genome^{203, 206}. When bacteriophage P1 DNA is integrated into a host cell genome, it is flanked by recombination signal sequences known as *loxP* sites. These sites are recognized by the enzyme Cre recombinase, which excises intervening DNA by cutting it at the sites and joining the two ends together to form a circle. By introducing *loxP* sites on either side of a gene or exon in the mouse genome, this mechanism can be used to excise specific genes or exons, and the process can be manipulated for cell specific deletion. Mice containing *loxP* sites flanking a specific gene or exons are mated with transgenic mice that express Cre recombinase under the control of a tissue-specific promoter. Therefore, the active Cre recombinase in the appropriate tissue will excise the DNA between the inserted *loxP* sites, effectively inactivating the targeted gene. Cells which do not express the promoter controlling Cre recombinase will not have excision of the relevant gene, resulting in a tissue specific knockout mouse.

1.10.1 T-cell specific IL-4R α -/- (Lck^{cre}IL-4R α ^{-lox}) mice

Mice with a specific deletion of IL-4R α on T-cells have been generated using the principles outlined above²⁰⁷ (Fig. 1.11). First, an IL-4R α -/- mouse²⁰⁵ was crossed with a transgenic mouse expressing cre recombinase under control of the T-cell specific

promoter, lymphocyte specific tyrosine kinase (Lck^{cre})²⁰⁸ in order to produce a $Lck^{cre}IL-4R\alpha^{-/-}$ mouse. Subsequently, the $Lck^{cre}IL-4R\alpha^{-/-}$ mouse was crossed with a mouse bearing two loxed $IL-4R\alpha$ alleles (lox/lox) in order to produce $Lck^{cre}IL-4R\alpha^{-/lox}$ offspring. $IL-4R\alpha$ hemizyosity ($^{-/lox}$) increases the probability of Cre-mediated deletion of the loxed allele. $Lck^{cre}IL-4R\alpha^{-/lox}$ mice were back-crossed to BALB/c wildtypes for 9 generations to generate $Lck^{cre}IL-4R\alpha^{-/lox}$ mice with a BALB/c background²⁰⁷.

$Lck^{cre}IL-4R\alpha^{-/lox}$ mice were identified by PCR genotyping, and FACS analysis and real-time PCR were then used to confirm the specificity and efficiency of $IL-4R\alpha^{-/-}$ deletion on T-cells²⁰⁷. The results showed that $IL-4R\alpha$ was efficiently deleted on $CD3^+CD4^+$ cells but incompletely depleted on $CD3^+CD8^+$ cells and NK T-cells, and remained present on B cells, $\gamma\delta TCR^+CD3^+$ T-cells, dendritic cells and macrophages. The $Lck^{cre}IL-4R\alpha^{-/lox}$ mice are therefore effectively $CD4^+$ T-cell $IL-4R\alpha^{-/-}$ mice. Isolated $CD4^+$ T-cells from these mice do not proliferate in response to $IL-4$ but maintain proliferative responses to $IL-2$. In addition, Th2 differentiation is impaired in naïve $CD4^+$ T-cells from $Lck^{cre}IL-4R\alpha^{-/lox}$ mice, while Th1 differentiation is not. However, NO production by macrophages is inhibited by $IL-4$ and $IL-13$ in these mice, and antigen-induced IgE is present at only slightly reduced levels as compared to wild-type mice. This demonstrates the impairment in $Lck^{cre}IL-4R\alpha^{-/lox}$ mice of $IL-4R\alpha$ mediated functions in $CD4^+$ T-cells but not in other cells such as macrophages and B-cells.

1.10.2 Macrophage/neutrophil specific $IL-4R\alpha^{-/-}$ ($LysM^{cre}IL-4R\alpha^{-/lox}$) mice

Macrophage/neutrophil specific $IL-4R\alpha^{-/-}$ mice have been recently generated¹¹⁹ (Fig. 1.12). Transgenic mice with a Lysozyme M promoter driving Cre recombinase were mated with $IL-4R\alpha^{-/-}$ mice to produce $LysM^{cre}IL-4R\alpha^{-/-}$ offspring. These offspring were mated with mice bearing $IL-4R\alpha$ flanked by loxp sites ($IL-4R\alpha^{lox/lox}$), resulting in offspring with LysM driven cre-mediated deletion of $IL-4R\alpha$ $LysM^{cre}IL-4R\alpha^{-/lox}$. PCR genotyping was used to identify $LysM^{cre}IL-4R\alpha^{-/lox}$ mice. Disruption of $IL-4R\alpha$ expression and function occurred in macrophages and neutrophils only, with functional tests demonstrating normal $IL-4/IL-13$ responsiveness in T-cells, B-cells and dendritic

cells. $LysMCreIL-4R\alpha^{-/lox}$ mice are therefore effectively macrophage/neutrophil specific IL-4R α knockouts. Accordingly, $LysMCreIL-4R\alpha^{-/lox}$ mice were found to have impaired IL-4/IL13 induced alternative activation of macrophages¹¹⁹.

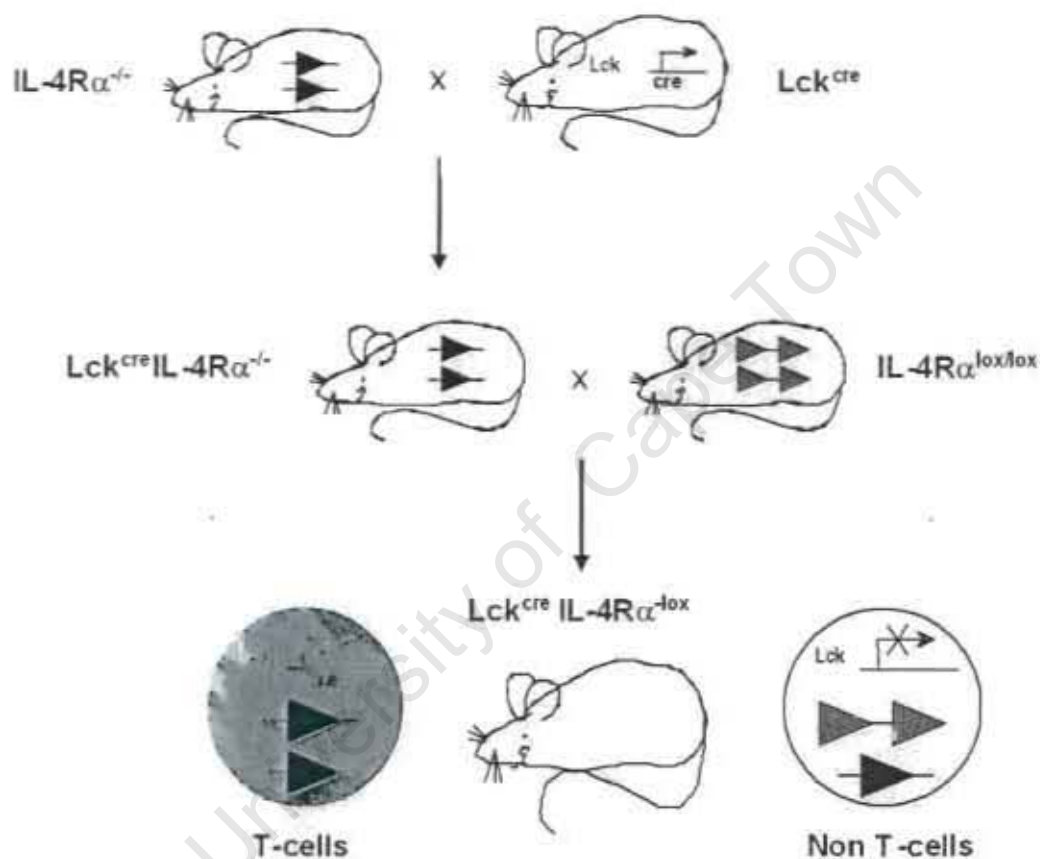


Figure 1.11. Generation of $Lck^{cre}IL-4R\alpha^{-lox}$ mice. Mice bearing Cre recombinase under control of a lymphocyte specific tyrosine kinase (Lck) promoter were mated with $IL-4R\alpha^{-/-}$ mice to produce $Lck^{cre}IL-4R\alpha^{-/-}$ mice. $Lck^{cre}IL-4R\alpha^{-/-}$ mice were subsequently mated with mice having $IL-4R\alpha$ flanked by loxp sites ($IL-4R\alpha^{lox/lox}$), resulting in offspring with Lck driven deletion of $IL-4R\alpha$ ($Lck^{cre}IL-4R\alpha^{-lox}$). Figure from Radwanska et al.²⁰⁷

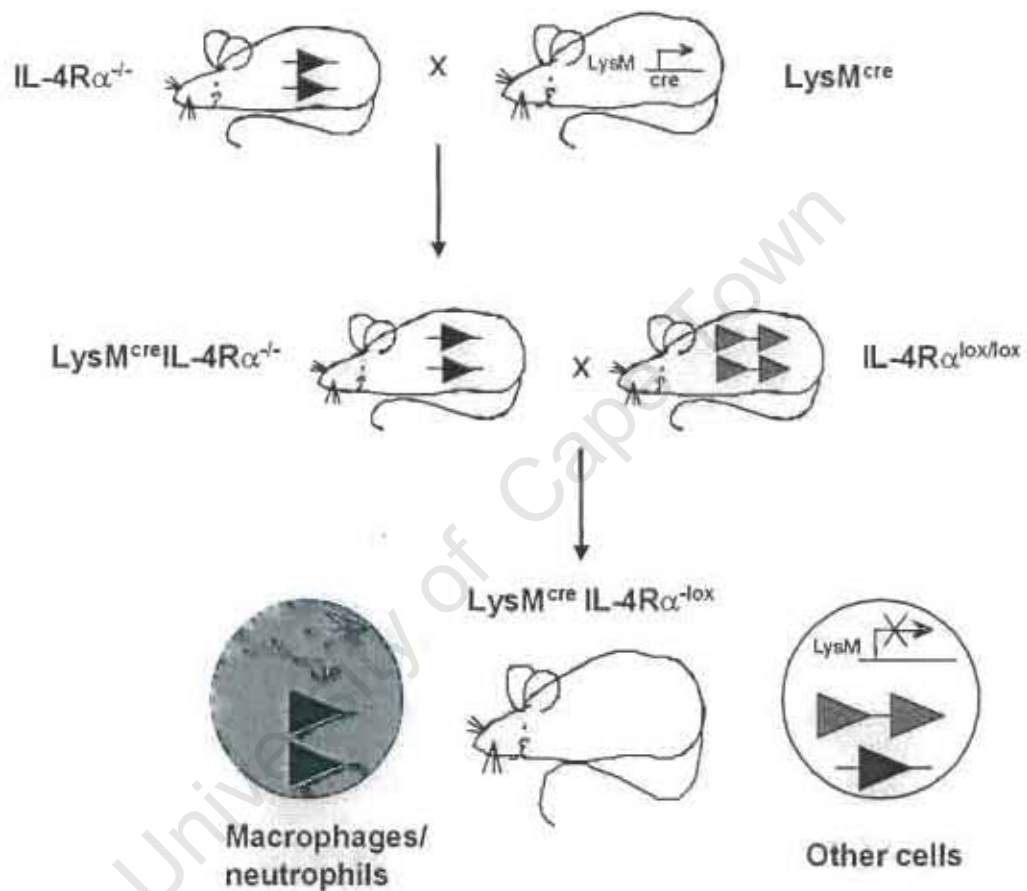


Figure 1.12. Generation of $LysM^{Cre}IL-4R\alpha^{-/lox}$ mice. $IL-4R\alpha^{-/-}$ mice were crossed with transgenic mice possessing a Lysozyme M promoter driving Cre recombinase, resulting in $LysM^{cre}IL-4R\alpha^{-/-}$ offspring. These offspring were mated with mice containing $IL-4R\alpha$ flanked by loxp sites ($IL-4R\alpha^{lox/lox}$) to produce mice with LysM driven cre-mediated deletion of $IL-4R\alpha$ ($LysM^{Cre}IL-4R\alpha^{-/lox}$). Figure adapted from Radwanska et al. ²⁰⁷.

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CHAPTER TWO

**IMMUNE AND ALLERGIC RESPONSES TO *ANISAKIS*
IN WILDTYPE AND GENE-DEFICIENT MICE**

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CHAPTER 2: Immune and allergic responses to *Anisakis* in wildtype and gene- deficient mice

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2.1 SUMMARY

Background: *Anisakis pegreffii*, a nematode that infests fish, is able to cause gastrointestinal disease and allergies in man. The present study examines immune and allergic responses against *A. pegreffii* in wildtype and gene deficient mice .

Methods: Wildtype, IL-4 $-/-$, IL-13 $-/-$ and IL-4 receptor alpha (IL-4R α) $-/-$ mice were injected intraperitoneally with live *A. pegreffii* larvae, and cellular responses were determined by analyzing infiltration of immune cells into the peritoneal cavity and peripheral blood eosinophilia. Cytokine and antibody responses were measured by ELISA and compared with responses generated by oral sensitization with *Anisakis* extract alone. Sensitized mice were challenged orally with *Anisakis* extract and examined for allergic reactions, histopathology and mast cell proteases.

Results: After *Anisakis* infection there was infiltration of immune cells into the intraperitoneal cavity in all strains, with IL-13 $-/-$ and IL-4R α $-/-$ showing the greatest infiltration along with splenomegaly. *Anisakis* infection induced a strong Th2/type 2 response in wildtype mice, typical of many gastrointestinal helminth infections. Importantly, when challenged orally with *Anisakis* extract, previously infected mice produced a response typical of food allergy, with itching, diarrhea and airway mucus hypersecretion. In contrast, allergic responses were reduced and abrogated in IL-4 $-/-$ and IL-4R α $-/-$ mice, respectively. Oral dosing of mice with *A. pegreffii* alone and no adjuvants resulted in production of type 2 antibodies IgG1 and IgE, but did not elicit strong Th2 responses, and little to no allergic response was seen after challenge.

Conclusions: These data show that *Anisakis* infection predisposes to allergic responses against ingested *Anisakis* proteins, and that both IL-4 and IL-13 are important in *Anisakis* allergy. Different immune responses to *Anisakis* in the various strains may represent the diverse responses to *Anisakis* infection in humans.

Table 2.1: Dishes at risk of harbouring infectious anisakid larvae

Country or region of origin	Dish
Japan	Sushi or sashimi
South America	Ceviche (raw fish marinated in lemon juice)
Spain	<i>boquèrones en vinagre</i> (anchovies in vinegar sauce)
Italy	<i>alici marinate</i> (pickled anchovies)
The Netherlands	Green herring (salted or smoked)
Germany	<i>rollmops</i> (pickled herring)
Scandinavia	<i>gravlax</i> (dry, cured salmon)
Hawaii	<i>lomi-lomi</i> (raw salmon)
South Africa	Smoked snoek or undercooked cater (snoek roe)
U.K.	Smoked salmon
Italy, U.K., U.S.A.	Seared tuna
Russia	Raw caviar/fish roe
Malaysia	Fish tripe (undercooked)
Worldwide	Undercooked fresh fish, e.g. hake, cod
Worldwide	Raw or undercooked squid, octopus, molluscs and crustaceans

2.1 INTRODUCTION

2.2.1 Anisakiasis

Anisakiasis refers to the disease caused by ingestion of members of the anisakidae family, which includes *Anisakis* spp, *Pseudoterranova* spp., *Contracaecum* spp and *Hysterothylacium* spp.¹. The source of infectious larvae causing anisakiasis is commonly a dish consisting of raw, pickled or smoked fish, examples of which are to be found in many countries (see Table 2.1). Accordingly, the incidence of anisakiasis is highest in countries where these dishes are frequently consumed. For example, in Japan, over 2000 cases of anisakiasis are reported every year². However, many infections have also occurred after consumption of freshly caught fish such as hake or cod that appears to be well-cooked but has not been sufficiently heated through (e.g. grilled, microwaved or shallow-fried fish)³.

Ideally, fish should be eviscerated immediately after being caught so that the large number of dormant larvae in the gut and mesenteries of the fish do not crawl into the flesh upon warming up⁴. In order to kill the smaller amount of larvae that may be present in the musculature, fish should be deep-frozen (-20°C for 24-48hrs hours) or cooked so that all parts of the fish reach at least 60°C for 10-20 minutes⁵⁻⁷. However, freezing or heating can alter the flavour of sushi or other raw fish delicacies^{7,8}. In Spain, an alternative method of pickling fish has been proposed in which the fish is exposed to high concentrations of acetic acid in order to kill any contaminating parasites, and the acid is subsequently diluted down for consumption⁹. Other seafoods such as squid and molluscs have also been implicated in *Anisakis* infections, and care should be taken to properly prepare these as well¹⁰.

In Japan and Europe, the majority (over 90%) of anisakiasis cases are caused by *Anisakis* species, followed by *Pseudoterranova decipiens* (approximately 4-5%) and then other unspecified species, possibly *Contracaecum* or *Hysterothylacium*^{1, 7, 11}. However, these figures vary from place to place due to differing geographical distribution of species, with *Pseudoterranova* causing anything from 0-22% of anisakiasis in various regions of

Japan¹², and often reported as the cause of infections in the Americas¹³⁻¹⁵. Little epidemiological information is available from Africa. Reported cases include two children from Egypt with urticaria due to *Anisakis* sensitization¹⁶ and a South African man with food-related allergic reactions due to *Anisakis*¹⁷. In addition, a study of occupational allergies amongst seafood-processing workers in the Western Cape of South Africa found a prevalence of 8% sensitization to *Anisakis*¹⁸, and additional cases of occupational *Anisakis* allergy are occasionally reported to our unit. Anisakiasis or discovery of anisakine larvae in patients has not been reported from South Africa, although presumably cases of live infection do occur, especially with the recent trend towards visiting sushi restaurants, and the high levels of *Anisakis* infestation in the fish snoek (*Thyrsites atun*), a local favourite that is commonly sold fresh (i.e., unfrozen).

Infection with *Pseudoterranova* spp, *Contracaecum* spp. or *Hysterothylacium* spp. is often mild and transient, causing nausea, cramps, pharyngeal irritation, and occasionally allergic symptoms^{14, 15}. Larvae are usually coughed or vomited up 36 hours to 2 weeks after ingestion. Occasionally, especially in the case of *Pseudoterranova* spp, they can penetrate the gastric tract to cause acute disease, but on the whole are less invasive than nematodes of the species *Anisakis*^{12, 13}. Anisakiasis caused by infection with *Anisakis* spp. exists in gastric, intestinal and more rarely, extragastrointestinal forms, and may be acute or chronic. Infection with one or two worms is usual, but multiple infection is occasionally reported, and as many as 56 larvae have been found in one patient^{2, 19}. Symptoms of acute anisakiasis normally begin several hours to several days after ingestion of the live larvae.

2.2.2 Gastric anisakiasis

Typically, gastric infestations present within the first 12 hours after fish ingestion. In Japan, gastric anisakiasis is by far the most common form, whereas in Europe intestinal anisakiasis is said to be more frequent^{8, 11}. The reason for this discrepancy is not known, though it has been suggested that differences in food preparation may play a role¹¹. It seems more likely that differences in diagnosis or reporting are to blame⁵. In Japan there was once the misconception that intestinal anisakiasis was more common in the north and

gastric anisakiasis in the south, but it is now known that there is no difference in their distribution, and that the discrepancies were due to differences in utilization of gastrointestinal endoscopy⁷. In Europe, where there is less awareness of anisakiasis than in Japan, it is more likely to be misdiagnosed. Gastric anisakiasis can be confused with food poisoning, indigestion, gastric ulcer or appendicitis, and can spontaneously resolve if the larvae are vomited up during the acute stage.

Symptoms of gastric anisakiasis can include acute abdominal pain (mild to severe), nausea, vomiting, diarrhoea, abdominal distention, chest pain, urticaria and anorexia, though in some patients the initial infection is asymptomatic and is only discovered subsequently when the disease becomes chronic or larvae are discovered accidentally during surgery^{20, 21}. After ingestion, *Anisakis* larvae secrete proteases that enable them to penetrate the mucosal wall, eliciting an inflammatory response²². Endoscopy during acute gastric anisakiasis reveals reddening of the gastric mucosa, oedema, haemorrhage and ulcerations or erosive lesions at the site of larval penetration, while radiology shows thickening of gastric folds, and may reveal the larva itself^{23, 24}. Gastrosopic removal of the larva(e) with forceps brings rapid relief²⁵. If pain is tolerable, treatment with antihelminthics, anti-inflammatories and analgesics may be sufficient²⁶⁻²⁹. However, if the larvae are not removed, treated or naturally expelled, they may penetrate into the submucosa and elicit eosinophilic infiltration and granuloma formation, causing symptoms of chronic gastric anisakiasis, including bloating, loss of appetite, dyspepsia and occasionally gastric bleeding. Symptoms may continue long after the larva has perished, due to a continuing inflammatory response to larval remains.

Chronic gastric anisakiasis may be confused with eosinophilic gastritis, Crohn's disease or gastric ulcer^{5, 30, 31}. Abscesses or submucosal tumour-like lumps can form, and several reports illustrate cases where gastric cancer was diagnosed, following which biopsy revealed remains of *Anisakis* larvae in pseudo tumours^{32, 33}. It has even been postulated that *Anisakis* infection is a risk factor for gastric cancer, due to its association with polyadenoma tumours and the *in-vitro* mutagenic and tumour-promoting, anti-apoptotic activity of certain fractions of *Anisakis* extract^{34, 35}. Japan simultaneously lays claim to

the highest incidence of gastric cancer and gastric anisakiasis in both men and women. In Chile, where raw fish is also frequently consumed as *ceviche*, gastric cancer is also very frequent³⁵.

2.2.3 Intestinal anisakiasis

Acute intestinal anisakiasis manifests as severe pain in the lower right quadrant of the abdomen, accompanied by nausea, diarrhoea, vomiting and fever, usually beginning 8-48 hours after ingestion of live larvae^{36,37}. It can be confused with acute appendicitis, ileitis or food poisoning^{27, 38}. Radiological findings show oedema, eosinophilic infiltration, thickening of the bowel wall and a resultant narrowing/obstruction of the intestinal tract^{21, 37, 39-41}. Leukocytosis with neutrophilia is common and occasionally eosinophilia may occur^{42, 43}. In cases where pain is intolerable, surgical resection is required to remove the worm. Otherwise, a combination of anti-parasitic and anti-inflammatory drugs may suffice, as in gastric anisakiasis. However as in the case of gastric anisakiasis, if acute intestinal anisakiasis is not treated, chronic intestinal anisakiasis may result, with deposition of connective tissue and accumulation of eosinophils around larval remains in the submucosa^{1, 30}. Persistent cramps in the lower abdomen, intermittent constipation and diarrhoea, weight loss and sometimes bloody stools can result from the inflammation and/or intestinal obstruction^{21, 27, 36, 44}. These symptoms can persist for years until the granulomas and larval remains are removed by surgery. Often, such symptoms are mistaken for Crohn's disease, ulcerative colitis, diverticulitis, appendicitis, non-specific eosinophilic enteritis or irritable bowel syndrome²⁷.

2.2.4 Extragastrintestinal (ectopic) anisakiasis

More rarely, after penetrating the mucosa the *Anisakis* larva may migrate to extragastrintestinal locations. Larvae have been found in such places as the oral cavity, spleen, lungs, oesophagus, pancreas, ovaries, peritoneum, liver and lymph nodes^{7, 35, 45-48}. They may elicit inflammatory reactions that lead to their discovery, or encapsulate and remain there until being gradually degraded⁴⁹. Occasionally they are found accidentally during surgery. A Japanese study found an incidence rate of 0.4% (52 of 14162 cases) for ectopic anisakiasis.

2.2.5 Gastroallergic anisakiasis

In a subset of patients, *Anisakis* infection is accompanied by allergic symptoms such as urticaria, angioedema, bronchospasm and anaphylaxis^{43, 50, 51}. The ensuing combination of gastrointestinal and allergic symptoms has been termed “gastroallergic anisakiasis”⁴³. Sera from patients with gastroallergic anisakiasis typically already show *Anisakis*-specific IgE at the time of the reaction, as opposed to sera from patients with gastric anisakiasis, in which specific antibodies may only appear 10-30 days after infection^{35, 52}. It has therefore been speculated that gastroallergic anisakiasis is a memory response to a prior, possibly asymptomatic infection in conjunction with a primary response to previously unrecognized antigens⁵³. Hypersensitivity symptoms may precede, follow, or coincide with gastrointestinal symptoms, and tend to dominate the clinical picture. Often the severe epigastric pain and mucosal oedema common to gastric anisakiasis are not present.

Symptoms can begin anywhere between a few hours to more than a day after ingestion of the parasite, and patients may therefore not relate the ingestion of the fish to the symptoms. The disease appears to be short-lived, with symptoms of some patients disappearing before arrival at the emergency room. Most recover within one day after treatment with epinephrine, corticosteroids and antihistamines, and removal of the larvae if present. Daschner et al 2002⁵³ suggest that the allergic reaction against the parasite helps to expel it more quickly, explaining the generally shorter course of the disease as compared to gastric or intestinal anisakiasis. However, because a high proportion of the reactions are anaphylactic and therefore life-threatening, some patients do require longer periods of hospitalization.

Studies in countries with a high consumption of fish indicate that a large proportion of anaphylaxis cases are due to *Anisakis*. In Spain, when considered a hidden food allergen, *Anisakis* is responsible for the largest number of anaphylactic reactions to food^{51, 54}. An analysis of 665 cases of anaphylaxis at a Spanish hospital found that *Anisakis* was responsible for more cases of anaphylaxis than all foods combined, and was the third most common cause of anaphylaxis, following drugs and *Hymenoptera* stings⁵⁵.

Ingestion of *Anisakis* is also an important cause of chronic urticaria in these countries^{54, 56-58}. Because larvae are not always found in patients diagnosed with gastroallergic anisakiasis or urticaria, it is uncertain whether a live worm is required in order to elicit the allergic symptoms or whether allergic reactions can occur to dead parasites or their proteins. Daschner et al⁵⁹ consider pure allergic reactions to *Anisakis* without gastrointestinal symptoms to be rare. Several oral challenge studies found that patients tolerated the ingestion of capsules containing killed *Anisakis* larvae and were symptom free on a diet allowing deep-frozen fish⁶⁰⁻⁶². However, several other studies note that some patients have *Anisakis*-related reactions to well-cooked fish, including canned tuna, and a small number of patients on a deep-frozen fish diet continued to experience allergic reactions^{54, 56, 63-65}. Furthermore, several proteins found in *Anisakis* are both heat stable and resistant to pepsin digestion^{64, 66-69}. A case has also been reported where a patient had severe anaphylactic reactions to a skin prick test with *Anisakis* extract on two occasions⁷⁰. Whether or not live larvae are required for allergic reactions is therefore still controversial⁵.

2.2.6 Diagnosis of anisakiasis

In the past anisakiasis has often been misdiagnosed as appendicitis, ileitis, Crohn's disease, eosinophilic gastroenteritis, gastric ulcer, food poisoning, fish allergy, idiopathic anaphylaxis, idiopathic urticaria and even gastric or intestinal cancer^{27, 63}. However, in countries such as Japan, Spain and Italy where cases occur relatively frequently due to consumption of raw fish, diagnosis is now improving. In other parts of the world, it is likely that many cases of anisakiasis are still being missed.

Abdominal pain, nausea, vomiting and/or diarrhoea within 48 hours of consuming fresh seafood should indicate the possibility of *Anisakis* infection^{43, 63}. As many cases of anisakiasis have occurred after consumption of presumably well-cooked hake or cod, ingestion of raw seafood should not be the only factor meriting further investigation. Discovery of larvae is a definitive diagnosis, but usually only in gastric and colonic anisakiasis can they be directly seen by endoscopy^{35, 71} and in chronic cases they have

usually already passed through the mucosal wall⁵. Resected intestine can be histopathologically examined for larval remains⁵.

Radiography can reveal larvae appearing as thin filling-like defects²³. Pathology of the gut and intestine observed by radiology or ultrasonography contributes to the diagnosis but is non-specific^{23, 24, 30}, as is leukocytosis³⁷, and eosinophilia is only seen in a subset of patients^{35, 37, 42, 43, 72}. One study found that eosinophil cationic protein appears to be raised in the first 72 hours following infection and may therefore be a useful marker when combined with a history and symptoms⁴².

Positive skin prick tests to *Anisakis*, *Anisakis*-specific IgE and raised total IgE are good diagnostic markers during gastroallergic anisakiasis or chronic gastrointestinal anisakiasis^{27, 43, 73, 74}, but in primary infections serological conversion may take some time⁵³. Due to cross-reactivity of *Anisakis* somatic antigens with other helminths and invertebrates^{75, 76}, antibodies against excretory-secretory (ES) proteins may be better diagnostic markers⁶⁶, and a rise in specific and total IgE one month after the infection is considered a good indication of true infection⁴³. Antibodies against Ani s 1, an excretory protein of *Anisakis*, seem particularly specific to infection with live *Anisakis*^{77, 78}. An ELISA has been developed that utilizes this antigen⁷⁹, but it is not widely available. IgE immunoblotting analysis may also be used for diagnosis^{49, 64, 80}. When a hypersensitivity reaction dominates, as in gastroallergic anisakiasis, fish/seafood allergy should be ruled out by means of specific IgE determination or skin prick tests⁵.

2.2.7 Immunology of anisakiasis

The extent of tissue destruction and inflammation resulting from infection with *Anisakis* is out of proportion to the small size of the parasite (1-3cm long), indicating that host-parasite interactions are responsible for the pathology of anisakiasis¹¹. It is therefore important to understand immunological aspects of anisakiasis in order to fully comprehend the disease. Data on the immunological mechanisms behind *Anisakis*-associated pathology in humans are incomplete. Infection is primarily associated with intense eosinophilic infiltrates around the larvae, with some patients additionally showing

leukocytosis with increased neutrophils and others demonstrating peripheral blood eosinophilia. Studies in guinea pigs and rabbits demonstrated that *Anisakis* extract is strongly chemotactic for eosinophils but not neutrophils^{81, 82}. It is thought that tissue damage and not the worm itself evokes the neutrophils⁷². *Anisakis* extract injected intradermally into guinea pigs caused a large accumulation of eosinophils, starting within 1 hour and reaching a peak 8hrs after the injection. In patients, serum levels of eosinophil cationic protein (ECP) are raised in the first 72 hours after gastrointestinal infection⁴². Eosinophil Major Basic Protein (MBP), known to damage parasites, has been detected in eosinophils of the inflammatory infiltrate of biopsies from patients with anisakiasis⁸³. Inducible nitric oxide synthase (iNOS), another molecule that aids in parasite killing, was also expressed in the tissue and thought to be produced by the eosinophils. In vitro, *Anisakis* extract down-regulates NO production by macrophages⁸⁴.

The release of ECP, MBP and nitric oxide as well other products such as peroxidases and eosinophil-derived neurotoxin are probably responsible for worm death, but also cause local tissue damage⁷². The larvae usually die within a few days^{1, 72} and degenerate in about 8 weeks⁸⁵, during which time they are surrounded by necrosis, oedema and massive eosinophilia mixed with neutrophils, macrophages and lymphocytes^{7, 35}. A granuloma forms around the larval debris, with deposition of fibrotic tissue and formation of foreign body giant cell lymphocytes. In some patients this gradually disappears (leading to the term “vanishing tumours”^{7, 86}), while in others clearance can take longer and the continuing inflammation results in symptoms of chronic anisakiasis.

An examination by reverse-transcriptase PCR of intestinal resections from anisakiasis patients containing infiltrates of eosinophils and lymphocytes demonstrated expression of Th2 cytokines (IL-4, IL-5) and T cell receptor (TCR) in 100% of the tissues, but no IFN- γ or IL-2, indicating a Th2 type profile and suggesting that Th2 cells mediate the immunological effector mechanisms in anisakiasis⁸³. IL-4 stimulates IgE production, while IL-5 is responsible for inducing eosinophil proliferation, differentiation and activation⁸⁷. These data therefore fit with the features of the disease.

2.2.8 Animal models of anisakiasis

Several authors have used animal models of anisakiasis in an attempt to further elucidate the immunological mechanisms of disease⁸⁸⁻⁹⁵. The consensus is that intraperitoneal infection is the best route of inoculation, because in orally-infected mice more than 50% of the larvae can be lost via the anus, leading to problems with dose standardisation⁸⁹. When larvae that are not lost in this way reach the gastrointestinal lumen, they quickly burrow through the mucosa to enter the abdominal cavity, resulting in what amounts to intraperitoneal infection in any event. Jones et al have shown that the pathology of intraperitoneal infection closely mimics human infection⁸⁸. Furthermore, intraperitoneal injection is less traumatic for the mice than oral administration, due to the relatively large size of the larvae compared to the mice. Intraperitoneal infections were therefore used in the current study.

Jones et al (1990) surgically implanted *Anisakis* larvae into the abdominal cavity of CBA/J mice and looked at pathology at 1, 2 and 3 weeks post-infection⁸⁸. After one week, neutrophils had accumulated around the larvae. By two weeks, most larvae were still viable and surrounded by granulocytes, occasional multinucleate giant cells, and mature granulomata consisting of eosinophils, fibroblasts and collagen. At three weeks post infection the larvae had been invaded by inflammatory cells and were dead. Surrounding granulomata consisted primarily of connective tissue with scattered eosinophils, with multinucleate giant cells adjacent to the larvae and eosinophils adjacent to larval debris. Systemically mice had varying degrees of neutrophilia, which began to return to normal values by week 3. Peripheral blood eosinophils were slightly decreased at weeks 1 and 2, but also returned to normal by week 3, suggesting homing of the eosinophils to the site of the larvae during acute infection.

In a similar model using intraperitoneal injections with *Anisakis* larvae, Iglesias et al. measured antibody production during the first 8 weeks post infection in BALB/c x CBA/J mice⁸⁹. The majority of injected larvae were shown to remain viable up to 2 weeks, producing excretory-secretory (ES) products that stimulated an immune response. The larvae later died and were broken up, releasing internal components that induced a

secondary response. IgM and IgG1 were the predominant antibodies produced, with only slight increases in IgG2a, IgG2b, IgG3 and IgA. IgM antibodies against somatic antigens (SA) reached a peak at two weeks post infection, while IgG1 showed a first peak between 3 and 4 weeks post-infection and continued to climb up to 8 weeks post-infection. The authors suggest that the second peak was due to the release of new antigens during the break-down of the worm. Antibody responses against ES proteins peaked slightly sooner, with IgM at a maximum by week 1 and IgG1 peaking at week 3. Total IgG levels peaked for ES products at week 2 and SA at week 6, after which they declined and remained at a fairly constant level during the 21 weeks post-infection at which they were measured. Perteguer and Cuellar (2001) measured increases in IL-4 between days 6 and 12 and again at 3 weeks post oral infection, indicating the likely cause of the skewing towards type 2 antibody production ⁹¹. Studies in rats showed production of specific IgE that reached higher levels after a second infection, with a peak at 3 weeks after re-infection, indicating that an allergic state could be caused by multiple infections with *Anisakis* larvae ⁹⁴.

Different strains of mice have different responses to *Anisakis*, demonstrating the importance of the genetic background of the host in the immune response to *Anisakis* ⁹⁵. Human patients show a wide range of disease caused by *Anisakis*, ranging from asymptomatic to inflammatory to allergic. In this study, we aimed to determine the roles of the important Th2 type cytokines IL-4 and IL-13, as well as their receptor, IL-4R α , on the immune response to *Anisakis* infection and subsequent allergic responses. In order to do this we utilised gene-deficient mice with “knock-outs” in the genes for IL-4 ⁹⁶ or IL-4R α ⁹⁷ on a BALB/c background, and exposed them to intraperitoneal *Anisakis* infection. When available, IL-13 deficient mice ⁹⁸ were also included in the experiments. Antibody responses, pathology, and allergic responses were then compared to those of wild-type BALB/c mice.

2.3 METHODS

Mice

BALB/c mice (8-10-wk-old) and IL-4 ^{-/-} ⁹⁶, IL-13 ^{-/-} ⁹⁸ and IL-4R α ^{-/-} mice ⁹⁷ on a BALB/c background were housed under SPF barrier conditions using individual ventilated cages (IVC). All experiments complied with the South African Code of Practice, and were approved by the University of Cape Town's Animal Ethics Committee.

Anisakis larvae

Anisakis pegreffii larvae (L₃) were collected from the intestines and mesenteries of parasitized fish (*Thyrsites atun*) with forceps, and extensively washed in 4% acetic acid and phosphate-buffered saline (PBS)(see Appendix A). Larvae were frozen for extract or stored live at 4°C in Iscove's Modified Dulbecco's medium (IMDM) containing 100µg/ml penicillin and 100µg/ml streptomycin (all Gibco, Paisley, Scotland). Before use in live infections, larvae were re-washed in sterile PBS.

Anisakis extract

Anisakis larvae were frozen and homogenized in PBS, then sonicated with a MicrosonTM ultrasonic cell disrupter (Misonix)(Long Island, U.S.A.). The extract was centrifuged and filter-sterilized through a 0.20µm filter (Sartorius, Goettingen, Germany). Protein concentration was measured by BCA test (Pierce, Rockford, Illinois, U.S.A.). Concentrated extracts were produced by centrifugation through 15ml ultrafiltration units with a 10kD cut-off. (Millipore; Amicon, Cork, Ireland).

Anisakis infection

Mice were anaesthetized with 200µl of ketamine (Anaket-V; Centaur Labs, Johannesburg, South Africa)/xylazine (Rompun; Bayer, Isando, South Africa) anaesthetic (see Appendix A), and then injected intraperitoneally with two larvae in 1ml PBS (see Appendix A) at week 0 using a 12 gauge needle (Identipet, Pretoria, South Africa). In some experiments, mice were re-infected at week 8 with another two larvae.

Anisakis infection with oral challenge

Mice were anaesthetized with 200µl of ketamine (Anaket-V; Centaur Labs, Johannesburg, South Africa)/xylazine (Rompun; Bayer, Isando, South Africa) anaesthetic (see Appendix A), then injected intraperitoneally with two larvae at week 0, re-infected at week 8, and challenged orally with 5mg *Anisakis* protein extract at week 11 using oral dosing cannulae (VetTech, Cheshire, U.K.). Allergic reactions were evaluated using a modification of a previously described scoring system⁹⁹. 0, no symptoms; 1, scratching and rubbing around the nose and head, hypersensitivity to touch, irritability; 2, diarrhoea, puffiness around the eyes and mouth, pilar erecti, reduced activity; 3, cyanosis around mouth and tail, lying flat but upright; 4, lying on side, loss of grip, loss of consciousness; 5, death.

Oral sensitization protocol

Mice were dosed weekly with 100µl PBS or 1mg of *Anisakis* extract in 100µl PBS using oral dosing cannulae (VetTech, Cheshire, U.K.), for a total period of 5 weeks. On the 6th week mice were challenged orally with 5mg of *Anisakis* extract and observed for signs of allergic reactions (see above).

Peritoneal lavage

Mice were killed and intraperitoneal lavage was performed using 5ml of 10% FCS/PBS (Delta, Kemptom Park, South Africa). Cells were separated onto 3-aminopropyltriethoxysilane(APES)-coated slides by cytopspin, allowed to dry overnight and stained using a Shandon Kwik-DiffTM staining kit (Clinical Science Diagnostics, East Grinstead, U.K.). Differential counts were performed under a light microscope. Percentages were converted into cell numbers by multiplication with total cell counts.

Isolation of splenocytes

Spleens were aseptically removed from mice with forceps, and splenocytes were isolated by pressing through a metal sieve in 10ml IMDM(Gibco, Paisley, Scotland). The cells were centrifuged at 1200rpm for 5 minutes, and the pellet was resuspended in 3-5ml of

ice-cold red cell lysis buffer (Appendix A). After 2-5 minutes on ice, the cells were centrifuged at 1200rpm for 5 minutes. Pelleted cells were resuspended in 10ml of IMDM supplemented with 10% FCS (Delta, Kempton Park, South Africa), 2mM L-glutamine (Gibco, Paisley, Scotland), 100µg/ml penicillin (Gibco, Paisley, Scotland) and 100µg/ml streptomycin(Gibco, Paisley, Scotland) The splenocytes were then filtered through a 70µm cell strainer (BD Falcon, Bedford, Massachusetts, U.S.A.) to remove debris, and diluted 1:10 to 1:30 with Trypan Blue (Sigma, Steinheim, Germany) and PBS for counting.

Splenocyte restimulation

Splenocytes were diluted to 4×10^6 cells/ml with supplemented IMDM (Appendix A), and cultured in 48-well plates (Costar, Corning, New York, U.S.A.), with 250µl of cells added to one of the following: 250µl medium, wells pre-coated with anti-CD3 (clone 145-2C11; 10µg/ml) overnight at 4°C to which 250µl of medium had been added, 25µg conA in 250µl medium, 250µl of 200µg/ml *Anisakis* extract in medium, 250µl of 200µg/ml Grade V ovalbumin (Sigma, Steinheim, Germany) in medium. The cells were incubated at 37°C with 5% CO₂, and supernatants were collected after 48 hours and stored at -80°C.

Cytokine ELISAs

Sandwich ELISAs were performed to determine cytokine levels in cell supernatants. Nunc Maxisorp microtitre plates (Nunc, Reskilde, Denmark) were coated with purified anti-IL-4 (clone 11B11, 2µg/ml), anti-IFN-γ (clone An18KL6, 1µg/ml) or anti-IL-5 (1µg/ml), anti-IL-9 (2µg/ml), anti-IL-13 (1µg/ml), anti-IL-10 (1µg/ml) or anti-TGF-β (0.5µg/ml)(all BD Pharmingen) diluted in PBS, and incubated overnight at 4°C. Plates were blocked with ELISA block buffer (Appendix A) for 1 hour at 37°C, then washed 3x with ELISA wash buffer (Appendix A). Serially diluted standards (purified recombinant IL-4, IL-5, IL-9, IL-13, IFN-γ, IL-10 or TGF-β (all BD Biosciences Pharmingen, San Diego, U.S.A.) or cell supernatant, all diluted in ELISA dilution buffer (Appendix A) were added to the plates, and incubated overnight at 4°C. Samples to be used in the

detection of TGF- β were pre-treated with acid to unfold the protein for antibody binding. (Appendix B). The plates were washed 4x, and biotinylated goat-anti-mouse IL-4 (0.5 μ g/ml), IL-5 (0.5 μ g/ml), IL-9 (1 μ g/ml), IL-13 (0.5 μ g/ml), IFN- γ (0.5 μ g/ml), IL-10 (0.5 μ g/ml) or TGF- β (0.5 μ g/ml) antibodies (all BD Biosciences Pharmingen, San Diego, U.S.A.), diluted in ELISA dilution buffer, were added for 3 hours at 37°C. Plates were washed 4x and 1 μ g/ml alkaline phosphatase(AP)-labelled streptavidin (Pharmingen) was added for 1 hour at 37°C. Finally, plates were washed 4x and 4-nitrophenyl phosphate disodium salt hexahydrate (PNP) (Sigma, Steinheim, Germany) substrate (see Appendix A) was added. Absorbance was measured at 405nm with 492nm as a reference wavelength using a VERSAmax tuneable microplate reader (Molecular Devices, Sunnyvale, California, U.S.A.). Cytokine levels in supernatants were determined by reading from the standard curve.

Blood collection and eosinophil counts

Blood samples taken before challenge were collected in serum separator tubes (BD Microtainer™ SS7, BD, Franklin Lakes, U.S.A.) by tail vein bleeding under an infra-red heating lamp. Alternatively, blood was taken from killed mice after cervical dislocation. For eosinophil quantification, blood was diluted 1 in 10 in Discombe's fluid (Appendix A) and eosinophils were counted in a haemocytometer (Neubauer, Germany). For serum collection, blood samples were centrifuged for 15 minutes at 3000 rpm before being placed at -80°C for storage.

Antigen-specific antibody ELISAs

Nunc Maxisorp ELISA plates (Nunc, Reskilde Denmark) were coated overnight at 4°C with *Anisakis* extract in PBS at 5 μ g/ml (for IgG1, IgG2a, IgG2b) or 1mg/ml (for IgE), then blocked in ELISA block buffer (Appendix A) for 1 hour at 37°C. Plates were washed 3x with ELISA wash buffer (Appendix A), and serum samples serially diluted in ELISA dilution buffer (Appendix A) were added. Plates were incubated overnight at 4°C, then washed 4x. AP-labelled goat-anti-mouse IgG1, IgG2a, IgG2b or IgE, 0.5 μ g/ml, (Southern Biotechnology, Birmingham, Alabama, U.S.A.) was added for 2 hours at 37°C. Plates were washed 4x and PNP (Sigma, Steinheim, Germany) substrate (see Appendix

A) was added. Absorbance was measured at 405nm with 492nm as a reference wavelength, using a VERSAmax tuneable microplate reader (Molecular Devices, Sunnyvale, California, U.S.A.)

Total IgE ELISAs

Cytokine sandwich ELISA was performed using purified anti-IgE (clone 84.1C) as a coat (1µg/ml) and AP-labelled goat anti-mouse IgE (Southern Biotechnology, Birmingham, Alabama, U.S.A.) as a secondary antibody, according to the protocol outlined above ("Antigen-specific antibody ELISAs"). Purified recombinant mouse IgE (BD Pharmingen, San Diego, U.S.A) was used as a standard, starting at a concentration of 10µg/ml.

MMCP-1 and histamine ELISAs

MMCP-1 levels in serum were measured by ELISA according to the manufacturer's protocol (Moredun Scientific Ltd, Midlothian, U.K). Histamine levels were quantified using a histamine ELISA kit (IBL Immuno-Biological Laboratories, Hamburg, Germany).

Bronchiolar lavage

Mice were injected intraperitoneally with 300µl –1ml of lethal anaesthetic (Appendix A) as necessary. Dead mice were pinned to the dissecting board and sprayed with 70% ethanol. The trachea was exposed and the lungs were lavaged twice with 1ml of 10% FCS (Delta, Kempton Park, South Africa)/PBS using 1ml 18G cannulae (Introcan, B/Braun, Melsungen, Germany). The broncheolar lavage fluid was centrifuged at 2000rpm for 5 minutes and cells were resuspended in 500µl – 1ml of PBS. Viable cells were counted using Trypan blue (1:1 dilution)(Sigma, Steinheim, Germany). Cells were fixed to APES-coated slides using a cytopspin, and the slides were allowed to air dry overnight. Slides were stained using a Shandon Kwik-Diff™ staining kit (Clinical Science Diagnostics, East Grinstead, U.K.) and differential counts were performed using a light microscope.

Lung tissue analysis

Lungs were removed from killed mice, weighed, homogenized in 100mM Tris-Triton buffer (Appendix A), sonicated for 20 pulses at an output of 9 watts, and centrifuged for 20 minutes at 12000rpm. Supernatants were removed for eosinophil peroxidase (EPO) measurement. Alternatively, tissue was homogenized in PBS for antibody and MMCP-1 ELISAs. For histology, tissue samples were preserved in 4% phosphate-buffered formalin, cut in 5-7µm paraffin sections, and stained with haemotoxylin and eosin for cellular infiltration and Periodic acid-Schiff reagent for mucus.

EPO assays

Serial dilutions (1 in 2) of HRP standard (Sigma, Steinheim, Germany) were performed in a microtitre plate (Nunc, Reskilde, Denmark), using Tris-Triton buffer (Appendix A), with a starting concentration of 0.54 units. In parallel, lung tissue samples were serially diluted (1 in 10) in Tris-Triton buffer. The plates were incubated at 37°C for 10 minutes. Equal volumes of TMB substrate (KPL, Maryland, U.S.A.) were added and the plates were incubated at room temperature for approximately 2 minutes until the desired colour change was achieved. The reaction was stopped using equal volumes of 2M H₂SO₄, and the absorbance was measured at 450nm with a 540nm reference wavelength using a VERSAmax tuneable microtitre plate reader (Molecular Devices, Sunnyvale, California, U.S.A.).

Statistical analysis: Values are given as mean ± SEM or SD. Significant differences were determined using the unpaired two-tailed Student's *t*-test using computer software (GraphpadPrism™). For allergic score graphs, the Mann-Whitney test was used for two experimental groups, and the Kruskal-Wallis test with Dunn's post test for three or more groups. Values $P < 0.05$ were considered significant.

2.4 RESULTS

2.4.1 Cellular immune responses against *Anisakis*

Intraperitoneal injection of *Anisakis* larvae was associated with an influx of immune cells into the peritoneal cavity in all strains (Fig. 2.4.1). There was a steady rise in infiltrating cells after infection, with an increased number of cells at week 1 and a further increase by week 2. Larvae found in infected mice during the first two to three weeks after infection were usually still moving, while larvae found after this stage were dead. It was difficult to find larvae, but when they were discovered they were in most cases attached to the mesenteries or abdominal fat of the mice. There was no obvious effect of mouse strain on larval survival. However, the difficulty in finding larvae in these experiments precludes any definite conclusion about the effects of IL-4, IL-13 and the IL-4R α on destruction of larvae.

IL-4R α $-/-$ mice had higher numbers of infiltrating cells in the intraperitoneal cavity than other strains, followed by IL-13 $-/-$ and then IL-4 $-/-$ mice (Fig. 2.4.1). Wildtype BALB/c mice had the least inflammation, although the number of infiltrating cells did increase relative to sham-infected mice. The same pattern of inflammation was found in spleens, with increased numbers of splenocytes counted in spleens taken at week 2 in IL-13 $-/-$ and IL-4R α $-/-$ mice. Splenomegaly was especially evident in IL-4R α $-/-$ mice and was consistently observed over several experiments.

Differential cell counts showed a dramatic increase in the number of neutrophils in the intraperitoneal cavity on the day following infection (Fig. 2.4.2) in wildtype, IL-13 $-/-$ and IL-4R α $-/-$ mice. As IL-4 $-/-$ mice already had higher background levels of neutrophils, the increase was not noticeable in this strain. Eosinophils also began to appear at day 1, but were more evident from week 1 onwards, when their numbers had increased.

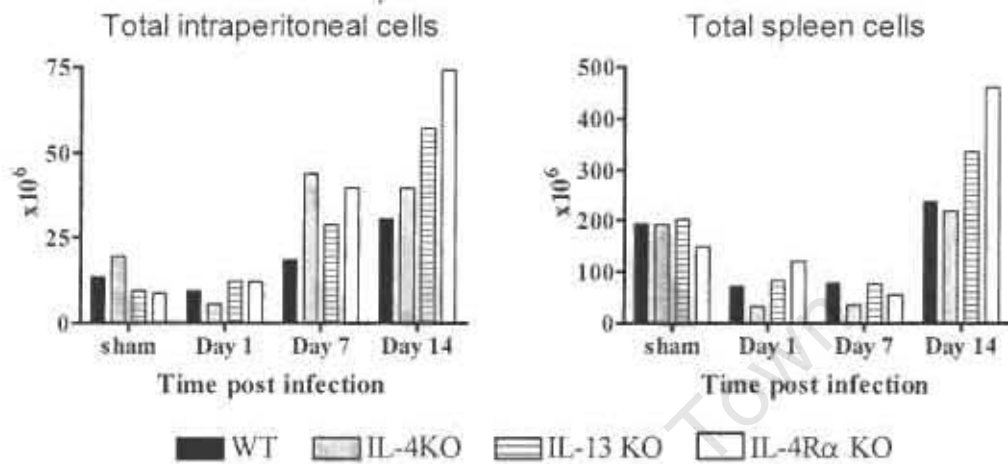


Figure 2.4.1 Local and systemic increases in immune cells following *Anisakis* infection. Cells from intraperitoneal lavages and spleens were pooled ($n = 4$) and counted following infection with 2 *Anisakis* larvae. Results are representative of at least two experiments.

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By week 1, increased numbers of monocytes/macrophages and lymphocytes were evident. Levels of all these cells remained increased at week 2 but had begun to drop back to normal levels at week 4. Mast cells were only present in very low numbers in the differential cell counts, but appeared to increase over time.

2.4.2 Peripheral blood eosinophilia after *Anisakis* infection

Absolute numbers of eosinophils in the peripheral blood varied between experiments and between male and female mice. However, there was a trend over several experiments towards an increase in peripheral blood eosinophils in wildtype, IL-4^{-/-} and IL-4R α ^{-/-} mice after infection, which reached significance in some of the experiments (Fig. 2.4.3). A peak seemed to occur at approximately three to four weeks post infection, around the time of death of the larvae. IL-13^{-/-} mice had only low numbers of eosinophils in the peripheral blood.

2.4.3 Cytokine production by peritoneal cells in *Anisakis* infection

Peritoneal lavage cells taken from sham-infected mice or at day 1 post infection did not produce significant amounts of cytokines when restimulated with *Anisakis* or anti-CD3 (Fig. 2.4.4). By week 1 post infection, splenocytes were producing cytokines, with wildtype mice producing predominantly Th2 cytokines and the knockouts showing mixed Th1/Th2 type responses. Similar responses occurred at week 2 post infection, although IFN- γ production had decreased. At week 4 post infection restimulation of peritoneal lavage cells with *Anisakis* extract led to production of IL-4, IL-5 and IL-13 in wildtype mice (Fig. 2.4.5). In IL-4^{-/-} mice, production of IL-13 was significantly lower and there was a trend towards lower IL-5 levels. Cells from IL-4R α ^{-/-} mice produced only low levels of cytokines.

2.4.4 CD4⁺ cells play a primary role in peritoneal IL-4 and IL-13 production

CD4⁺ cells were removed from peritoneal lavage cells using magnetic beads in order to investigate the role of CD4⁺ cells in cytokine production in the peritoneal cavity. Efficacy of CD4⁺ cell depletion was confirmed by FACS analysis (data not shown). Undepleted (total) peritoneal lavage cells from *Anisakis*-infected wildtype mice

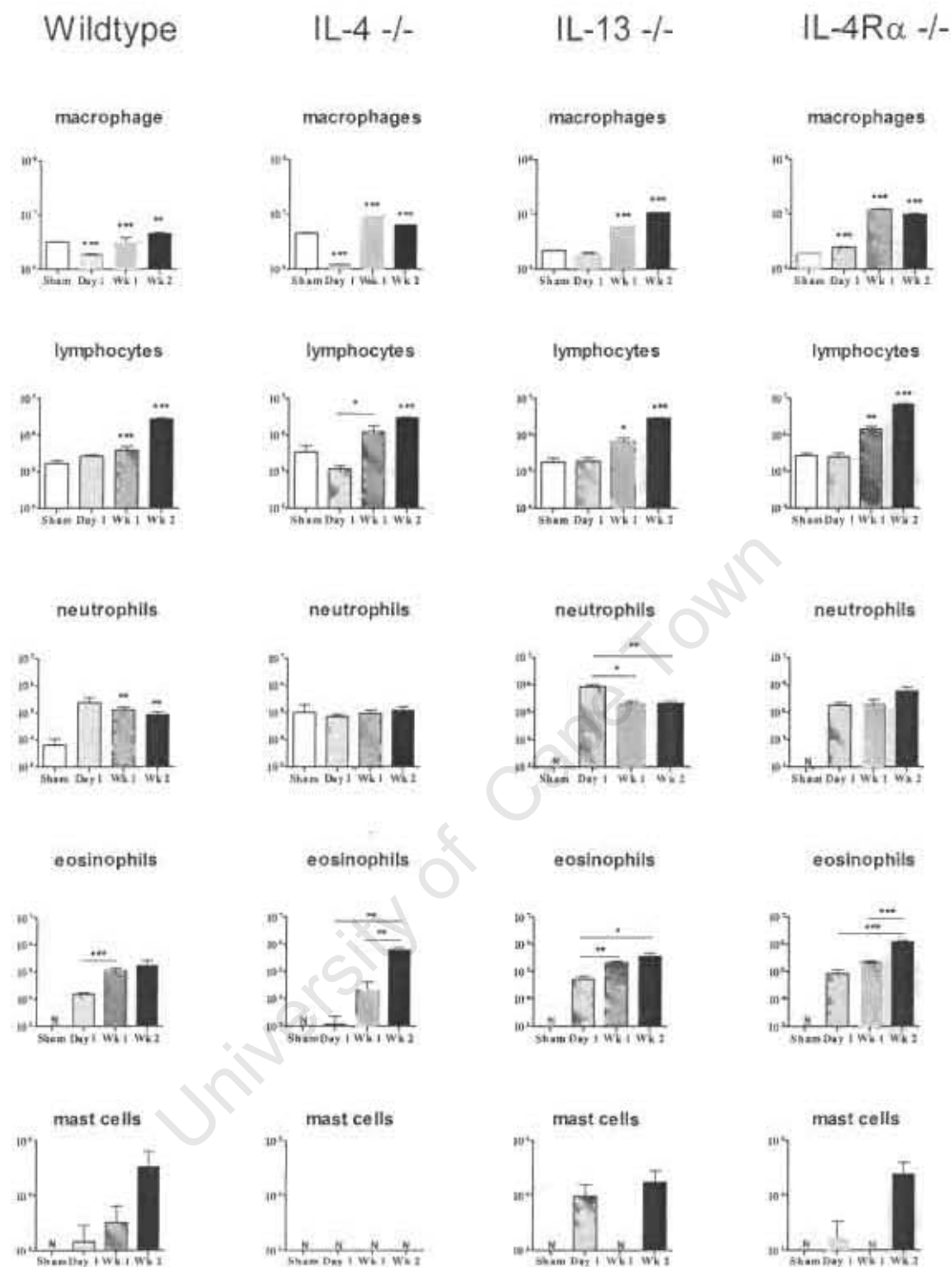


Figure 2.4.2. Differential cell counts of peritoneal lavage cells following *Anisakis* infection. Peritoneal cells from individual wildtype, IL-4^{-/-}, IL-13^{-/-} and IL-4R α ^{-/-} mice (n = 4) were fixed onto slides by cytopspin, and differential counts were performed. Data presented as mean \pm SEM. Statistical significance was assessed versus sham-infected mice. *, P<0.05; **, P<0.01; ***, P<0.001. N = not detected.

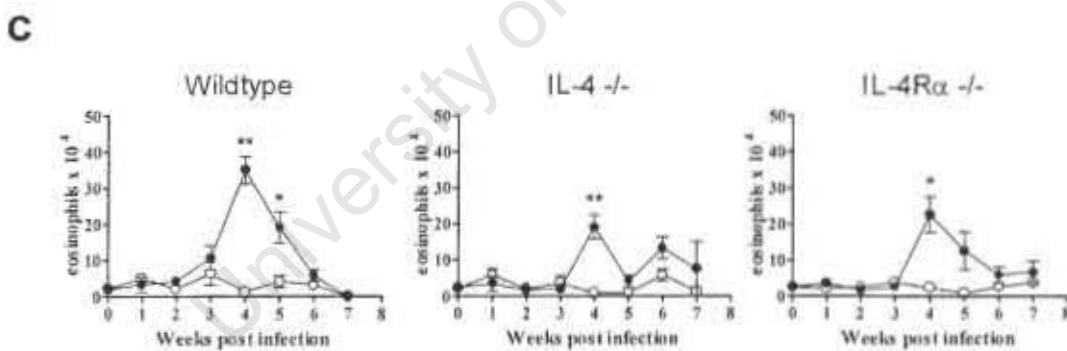
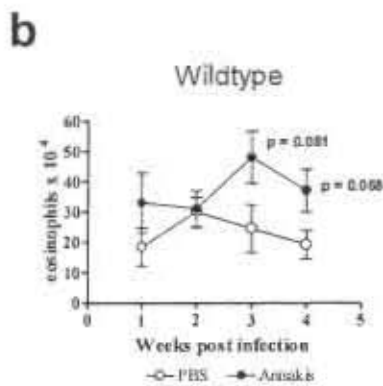
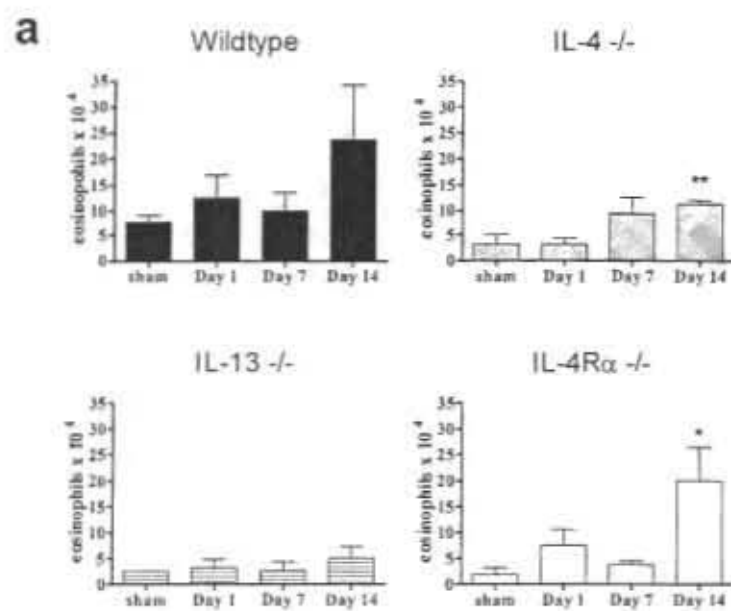


Figure 2.4.3. *Anisakis* infection resulted in a trend towards increased peripheral blood eosinophils. In three separate experiments (a-c) peripheral blood eosinophils were diluted in Discombe's fluid and counted following infection with 2 *Anisakis* larvae (n = 4-5). Data presented as mean \pm SEM. Statistical significance was assessed against sham-infected mice. *, $P < 0.05$; **, $P < 0.01$.

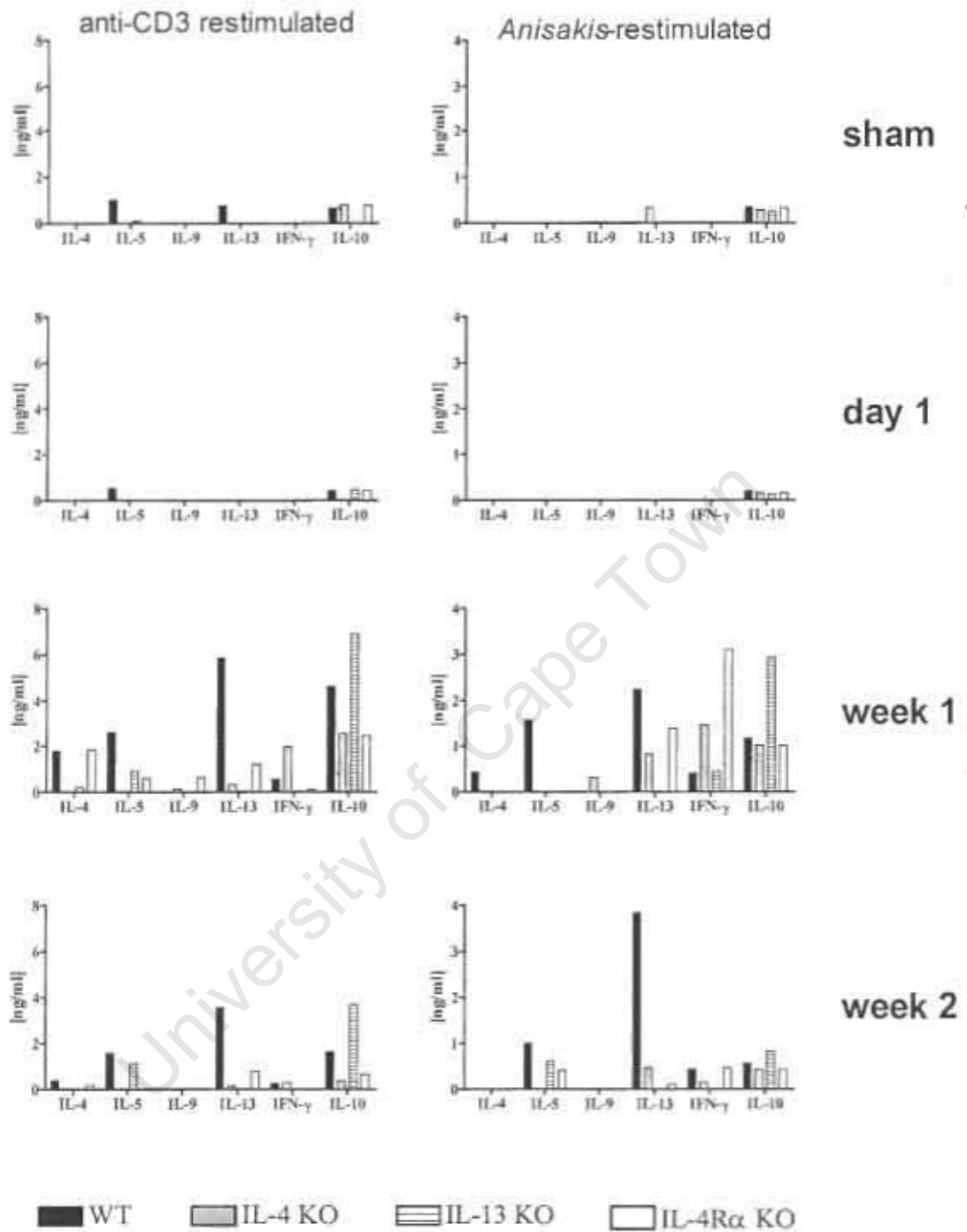


Figure 2.4.4. Peritoneal lavage cell cytokine production after infection. Pooled peritoneal lavage cells ($n = 4$) were restimulated with anti-CD3 or *Anisakis* extract, and cytokines in the supernate were measured by ELISA.

Anisakis-stimulated peritoneal lavage cells

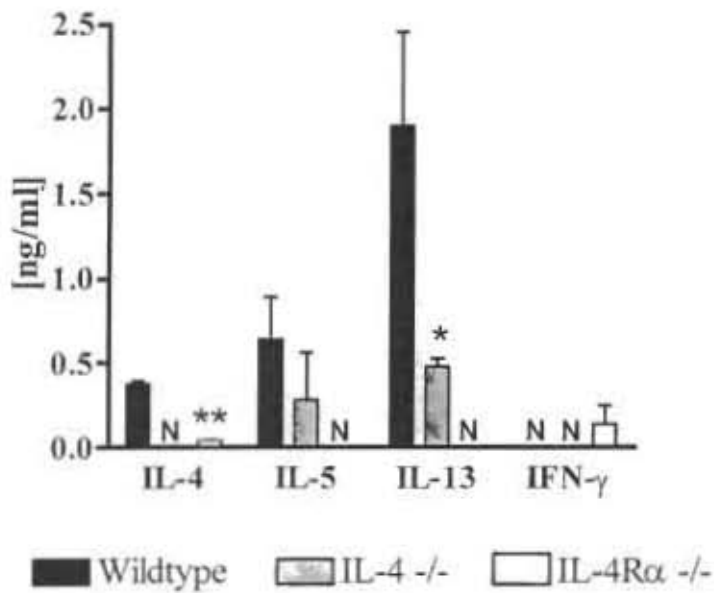


Figure 2.4.5. Cytokine production by peritoneal cells at week 4 post infection. Pooled peritoneal lavage cells (n = 4-5) isolated at week 4 post infection with 2 *Anisakis* larvae were restimulated with 100µg/ml of *Anisakis* extract. Results are representative of two experiments. Data presented as mean +/- SEM. Statistical significance was assessed between wildtype and knockout strains. N, not detectable; *, P<0.05; **, P<0.01.

restimulated with anti-CD3 demonstrated higher production of IL-4, IL-13 and IL-10 than sham-infected mice (Fig. 2.4.6). In contrast, CD4⁺ cell depleted peritoneal lavage cells had abrogated production of these cytokines. A decrease in IL-13 produced by *Anisakis*-stimulated peritoneal lavage cells was also observed following CD4⁺ cell depletion. This indicates that Th2 cells present in the peritoneal cavity following *Anisakis* infection are primarily responsible for production of IL-4 and IL-13. However, CD4⁺ cell depletion did not abrogate IL-10 production in *Anisakis*-stimulated peritoneal lavage cells. IL-10 may therefore be partially produced by non-CD4⁺ cells in response to *Anisakis*. Furthermore, CD4⁺ cell depletion abrogated IFN- γ production in peritoneal lavage cells from sham-infected, but not *Anisakis*-infected mice. It is likely that other infiltrating immune cells in *Anisakis*-infected mice are responsible for the remaining IFN- γ production. In addition, CD4⁺ cell depletion abrogated IL-5 production after anti-CD3 stimulation in sham-infected mice, but did not abrogate *Anisakis*-induced IL-5 production, indicating that *Anisakis* extract may directly stimulate non-CD4 cells to produce IL-5.

2.4.5 Splenocyte cytokine production in *Anisakis* infection

Splenocytes from wildtype, IL-4 ^{-/-}, IL-13 ^{-/-} and IL-4R α ^{-/-} mice were restimulated with anti-CD3 at various time-points post infection and cytokine responses were evaluated up till two weeks. Significant differences in patterns of cytokine production were observed among strains (Fig. 2.4.7-2.4.12). In wildtype mice, production of IL-4, IL-9 and IL-10 reached a peak between one day and two weeks post infection (Fig. 2.4.7 and 2.4.8). This was followed by a drop in production by week 2. Following this, IL-4 and IL-9 production began to rise again, perhaps as a result of release of proteins from killed larvae, and was significantly increased when evaluated at week 4 (Fig. 2.4.9 and 2.4.11). In contrast, IL-10 production remained at baseline levels. This could indicate that IL-10 production is stimulated by live larvae but not by the release of proteins by dead larvae. IL-13 and IL-5 production began to increase after week 1 and 2 respectively, perhaps as a consequence of the earlier peak in IL-4 production, and were significantly higher when measured later at week 4 (Fig. 2.4.7, 2.4.9 and 2.4.11). IFN- γ production was reduced up to two weeks post infection but by week 4 had returned to normal,

perhaps with resolution of the infection. At week 4, wildtype mice showed a cytokine profile that was significantly skewed towards a Th2 response (Fig. 2.4.9).

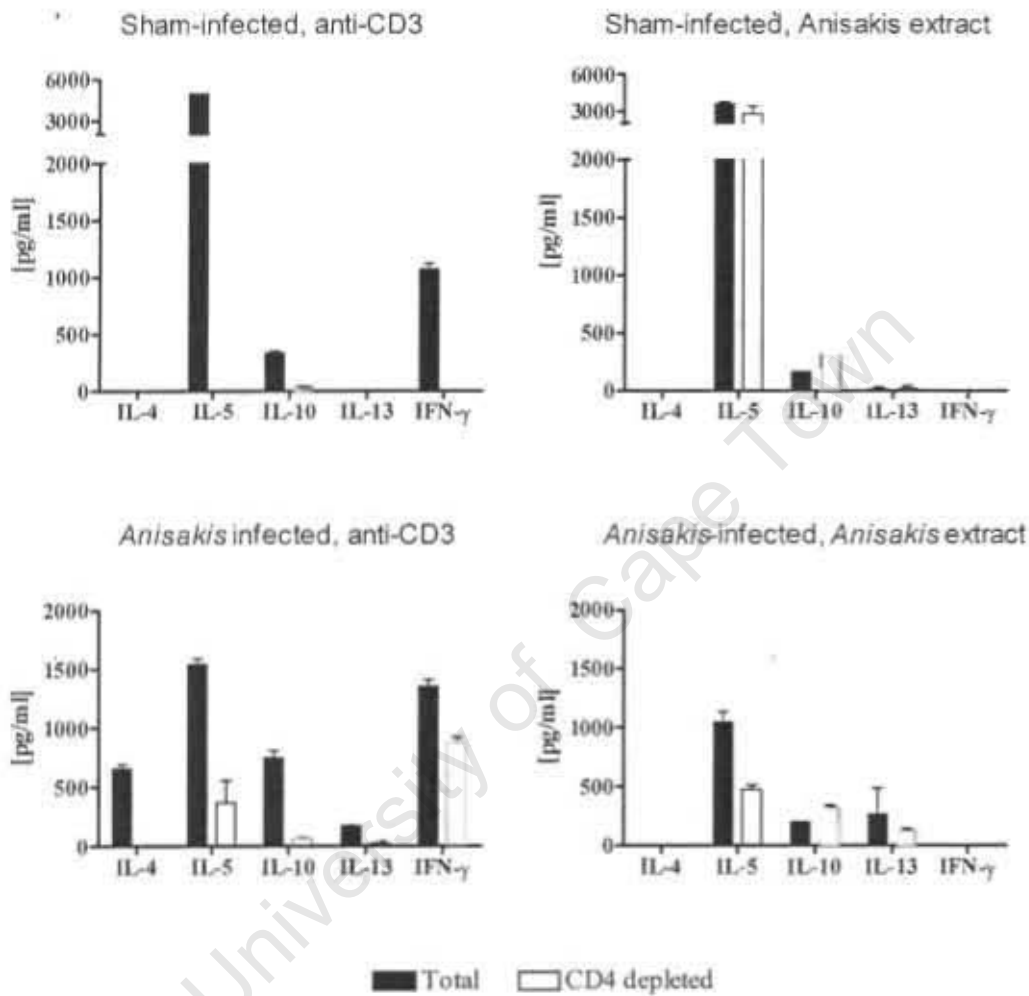


Figure 2.4.6. CD4⁺ cell depletion reduces cytokine production from peritoneal lavage cells. Peritoneal lavage cells taken at week 4 post infection (n = 4, pooled) were depleted of CD4⁺ cells using magnetic beads and restimulated with anti-CD3 or *Anisakis* extract in parallel with untreated cells (total peritoneal lavage cells). Cytokine production was measured by ELISA. Data presented as mean +/- SEM.

Like splenocytes from wildtype mice, IL-13 $-/-$ splenocytes showed increases in IL-4 and IL-9 production between day 1 and week 2, and there was an increase in IL-10 production at week 1 compared to day 1 and week 2 (Fig. 2.4.7). In addition, IFN- γ production by splenocytes dropped following infection (Fig. 2.4.8). However, IL-5 production showed a gradual decrease following infection.

In contrast, splenocytes from IL-4 $-/-$ mice did not show increased production of any of the Th2 cytokines, apart from a small and early increase in IL-9 (day 1). IFN- γ production was decreased after infection, as was IL-10 production. A similar pattern of cytokine production was found in IL-4R α $-/-$ mice, with a small initial peak in production of IL-9, unchanged IL-5, and decreased IL-13 and IFN- γ . An initial decrease in IL-10 production was followed by a slight but significant rise in production at week 2. In addition, IL-4 levels steadily increased after infection. When responses were evaluated at week 4, cytokine production had returned to normal in both knockout strains, apart from slightly raised IL-9 and slightly decreased IL-13 production in IL-4 $-/-$ and IL-4R α $-/-$ mice, respectively (Fig. 2.4.10 and 2.4.12).

2.4.6 Antibody responses in a primary *Anisakis* infection

Isotype specific responses were investigated in wildtype, IL-4 $-/-$ and IL-4R α $-/-$ mice (Fig. 2.4.13). Wildtype BALB/c mice produced predominantly type 2 antibodies in response to *Anisakis* larvae, with specific IgG1 and total and specific IgE. *Anisakis*-specific IgM levels started to rise within 1 week, peaking around week 2 and then declining slightly until week 5, when another rise was apparent. The second peak could have been a response to proteins released from killed larvae. IgG1 production commenced around sometime between week 1 and 2 and continued to rise. As with IgM, there was a sudden increase in IgG1 titres at week 5, reflecting a possible response to proteins released from destroyed *Anisakis* larvae. IgG1 titres remained at fairly constant levels until re-infection. Levels of IgG2a, IgG2b and IgG3 were extremely low, however there were small peaks at week 3 and 5. In contrast, IL-4 $-/-$ mice had raised levels of all isotypes except IgE. Again IgM was the first isotype present, raised at week 1 and remaining fairly constant with a slight peak at week 3, and a second peak at week 6.

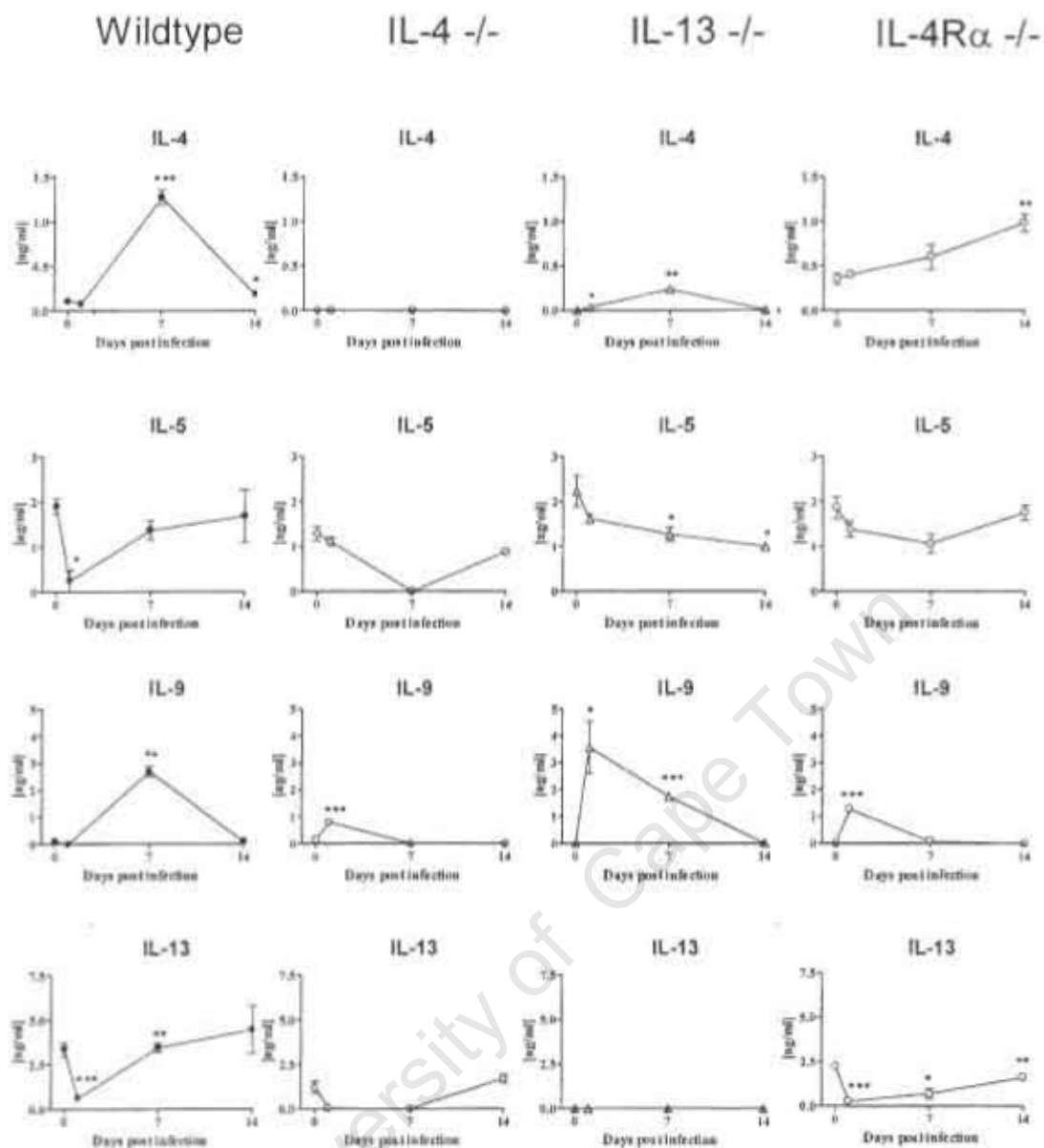


Figure 2.4.7. Kinetics of Th2 cytokine production from restimulated splenocytes. Splenocytes from sham-infected (represented on axis at day 0)(n = 4) and *Anisakis*-infected mice (n = 4) at 1, 7 and 14 days post infection were isolated and restimulated with anti-CD3. Cytokine production was measured by ELISA. Data presented as mean \pm SEM. Statistical significance versus sham-infected mice is shown. *, P<0.05; **, P<0.01; ***, P<0.001.

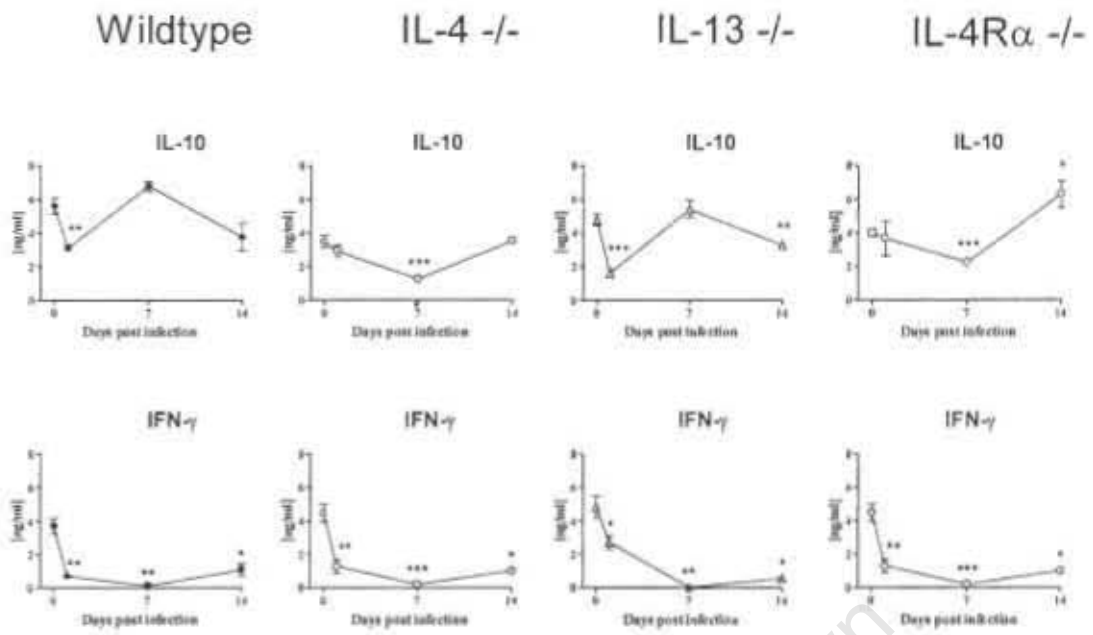


Figure 2.4.8. Kinetics of IFN- γ and IL-10 production from restimulated splenocytes. Splenocytes from sham-infected (represented on axis at day 0)(n = 4) and *Anisakis*-infected mice (n = 4) at 1, 7 and 14 days post infection were isolated and restimulated with anti-CD3. Cytokine production was measured by ELISA. Statistical significance versus sham-infected mice is shown. Data presented as mean +/- SEM.

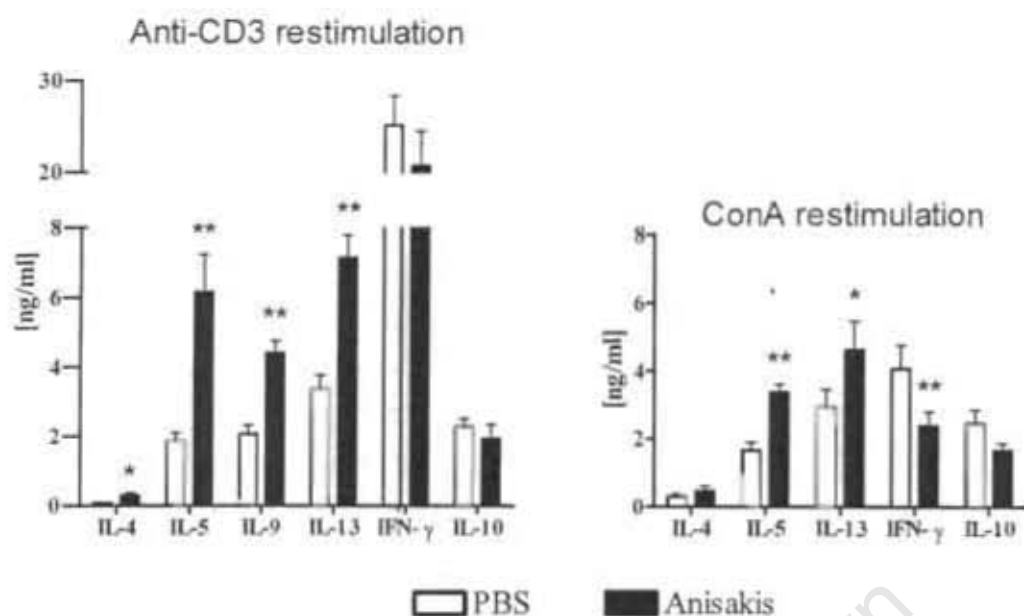


Figure 2.4.9. Cytokine production in wildtype mice at week 4. Splenocytes from individual mice infected with *Anisakis* or sham-infected controls (n = 5) were isolated at week 4 and restimulated with anti-CD3 or ConA. Cytokine production was measured by ELISA. There was not enough supernatant to test for IL-9 in conA restimulated cells. Data presented as mean \pm SEM. Statistical significance versus sham-infected mice is shown. *, P<0.05; **, P<0.01; ***, P<0.001.

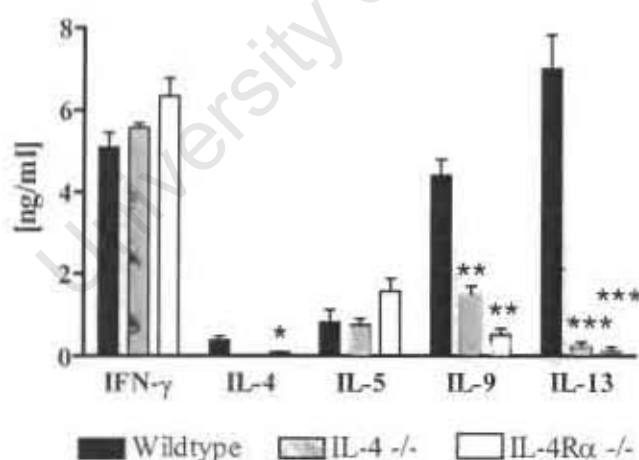


Figure 2.4.10. Cytokine production by wildtype, IL-4 $-/-$ and IL-4R α $-/-$ mice at week 4 post infection. Isolated splenocytes were restimulated with anti-CD3 and cytokine production was measured by ELISA. Data presented as mean \pm SEM. Statistical significance was assessed between wildtype and knockout mice. *, P<0.05; **, P<0.01; ***, P<0.001.

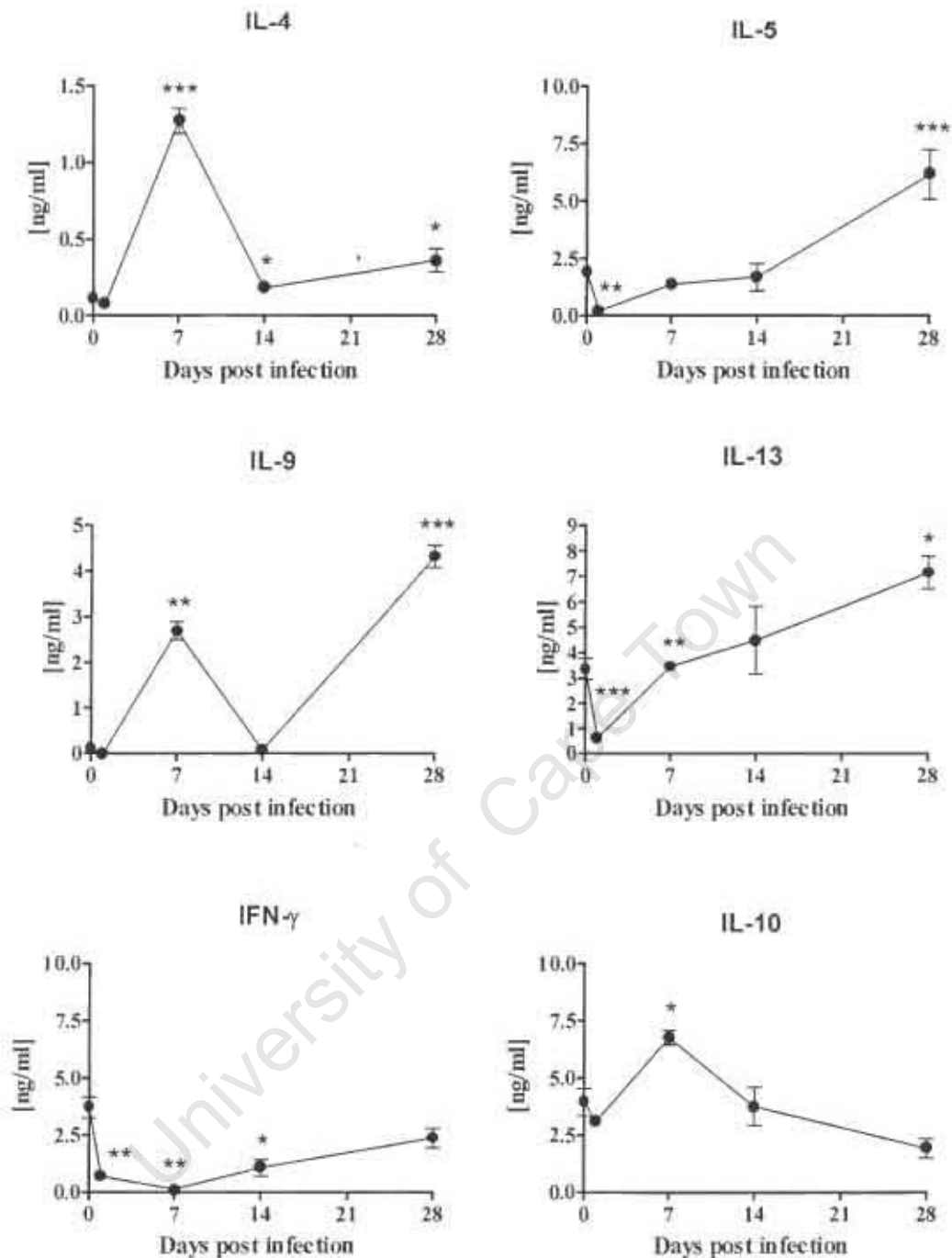


Figure 2.4.11. Th2 cytokines, but not IFN- γ or IL-10, are increased at week 4 in wildtype mice. Pooled splenocytes (n = 4) or individual splenocytes (week 4, n = 5) were restimulated with anti-CD3. Cytokine production was measured by ELISA. Data presented as mean \pm SEM. Statistical significance on comparison with sham-infected mice (day 0) is shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

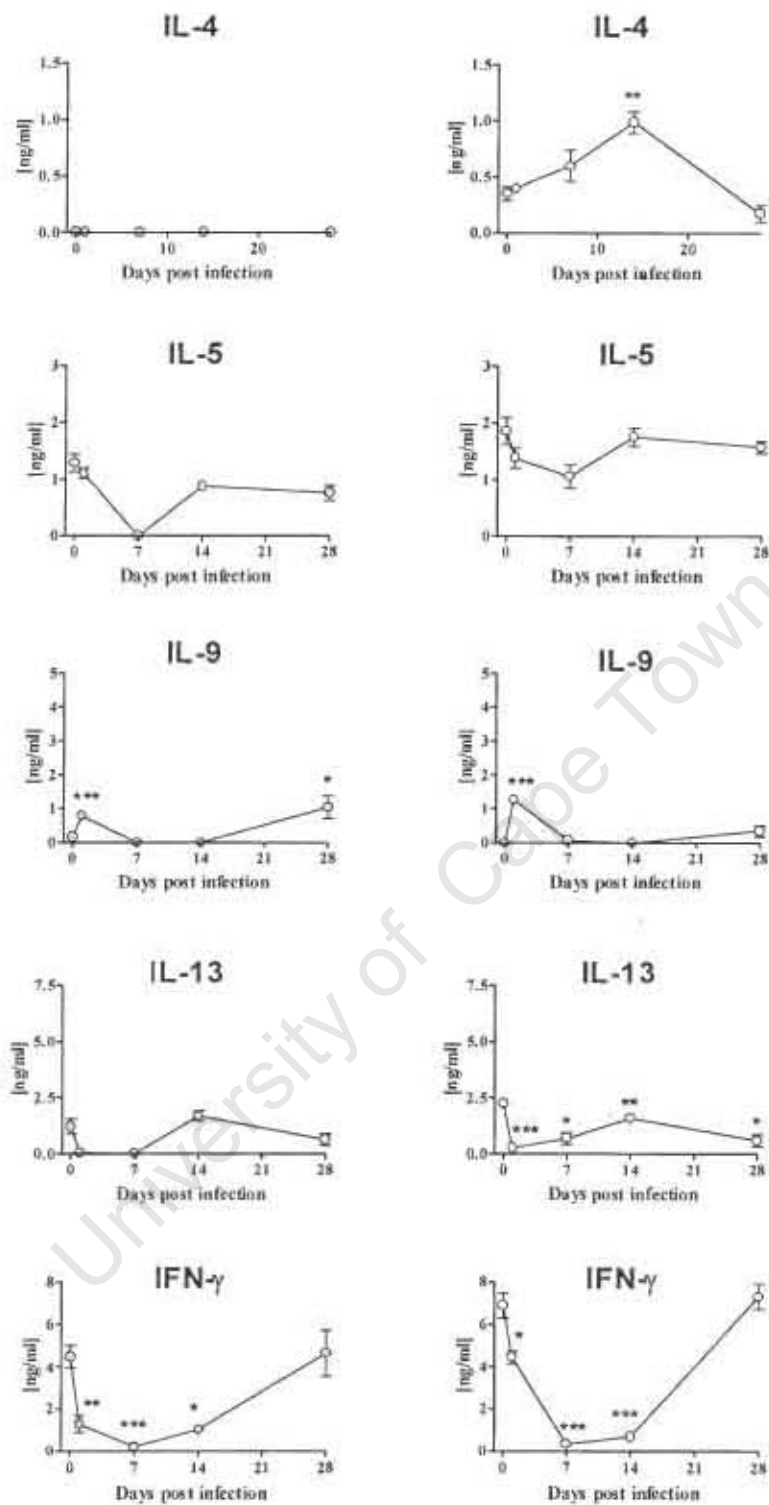


Figure 2.4.12. Kinetics of cytokine production in IL-4^{-/-} and IL-4Rα^{-/-} mice. Splenocytes were pooled (n = 4) and restimulated with anti-CD3. Data presented as mean +/- SEM. Statistical significance on comparison with sham-infected mice (day 0) is shown. *, P<0.05; **, P<0.01; ***, P<0.001.

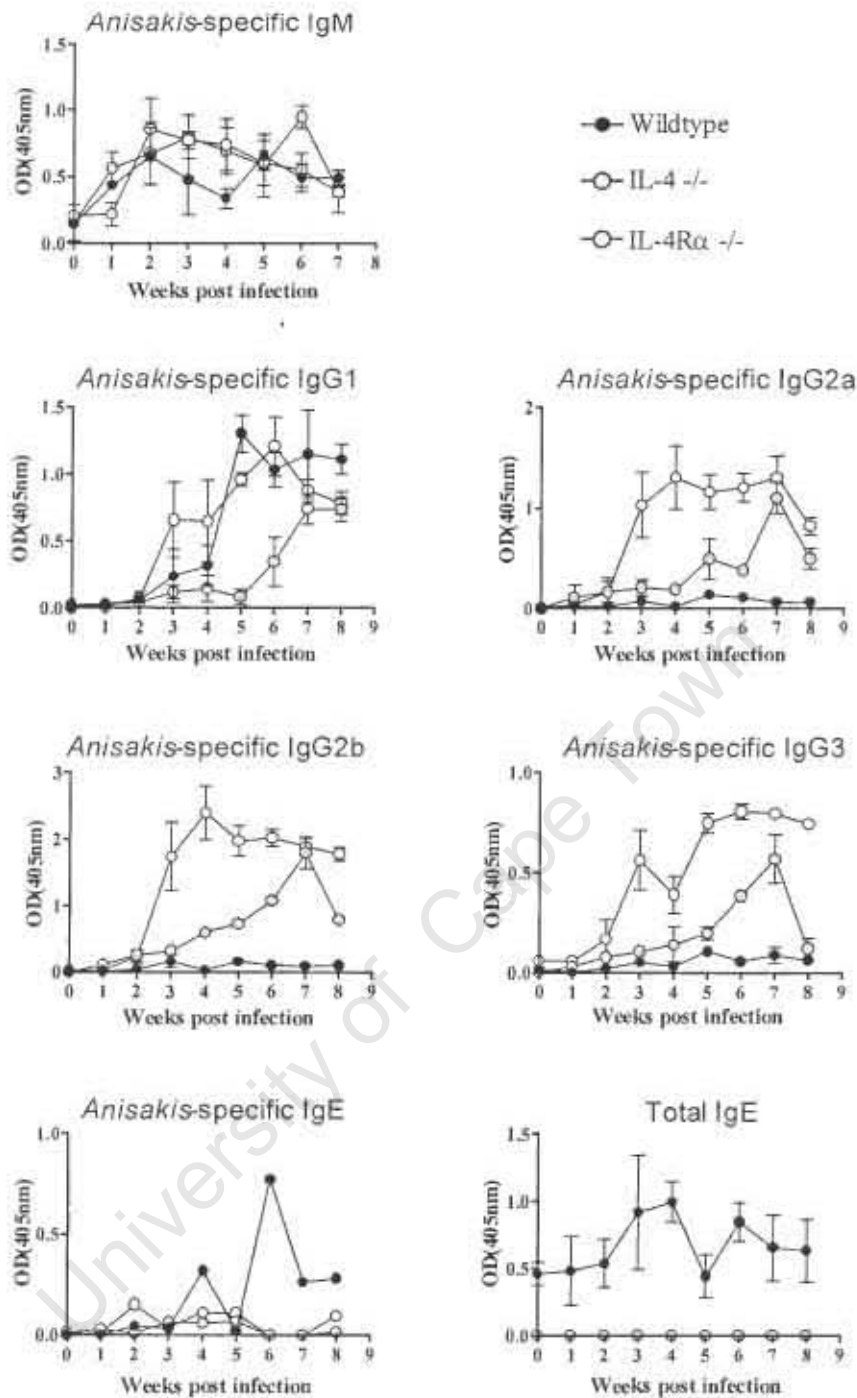


Figure 2.4.13. Antibody production after *Anisakis* infection. Antibody levels were measured in the serum of wildtype, IL-4^{-/-} and IL-4Rα^{-/-} mice post infection (n = 4). Results are representative of 2-3 experiments. Data presented as mean +/- SD.

Levels of all IgG isotypes began to rise at about week 2 post infection. IL-4R α $-/-$ mice produced even higher levels of all the IgG isotypes measured, commencing at about week 2. IgG2a and IgG2b levels remained fairly constant from about week 3 onwards, while IgG1 and IgG3 levels reached a second peak at week 6. IgM in this strain peaked at about week 2 post infection and then began to slowly decline. Total and specific IgE were not detectable in this strain.

IL-13 $-/-$ mice were not always available and a long-term study of antibody isotypes was therefore not performed in this strain. However, at week 5, IL-13 $-/-$ mice showed an intermediate profile compared to other strains. IgG1 was high, as in wildtype and IL-4R α $-/-$ mice, while IgG2a and IgG2b were lower than in IL-4 $-/-$ and IL-4R α $-/-$ mice but higher than in wildtype mice. Total IgE was present but at largely reduced levels compared to wild-type mice.

2.4.7 Antibody responses after re-infection with *Anisakis* larvae

Mice were re-infected with two live *Anisakis* larvae at week 8. After re-infection, wildtype mice showed an extremely strong memory response in terms of antibody production (Fig. 2.4.14), with a steep increase in IgG1 that was unparalleled by any increase in an IgG isotype in IL-4 $-/-$ or IL-4R α $-/-$ mice (Fig. 2.4.15). IgG1 levels peaked approximately 2 weeks after re-infection (week 10). This was accompanied by a rise in total IgE (peaking at approximately one week after re-infection, i.e. week 9) and a gradual and steady increase in specific IgE (Fig. 2.4.14). In IL-4 $-/-$ mice there was a gradual increase in all measured IgG isotypes after re-infection (IgG1, IgG2a, IgG2b, IgG3)(Fig. 2.4.15). In contrast, there was only a slight increase in IgG1 and IgG2a after re-infection in IL-4R α $-/-$ mice. Total IgE was extremely low or undetectable at all time-points measured in IL-4 $-/-$ and IL-4R α $-/-$ mice (Fig. 2.4.16). In one experiment specific IgE ELISAs indicated the presence of very low levels of *Anisakis*-specific IgE in IL-4 $-/-$ mice at three weeks post re-infection only. In other experiments specific IgE levels were below the detection limit in knockout strains, although occasionally an individual IL-4 $-/-$ mouse showed production of minute amounts of specific IgE.

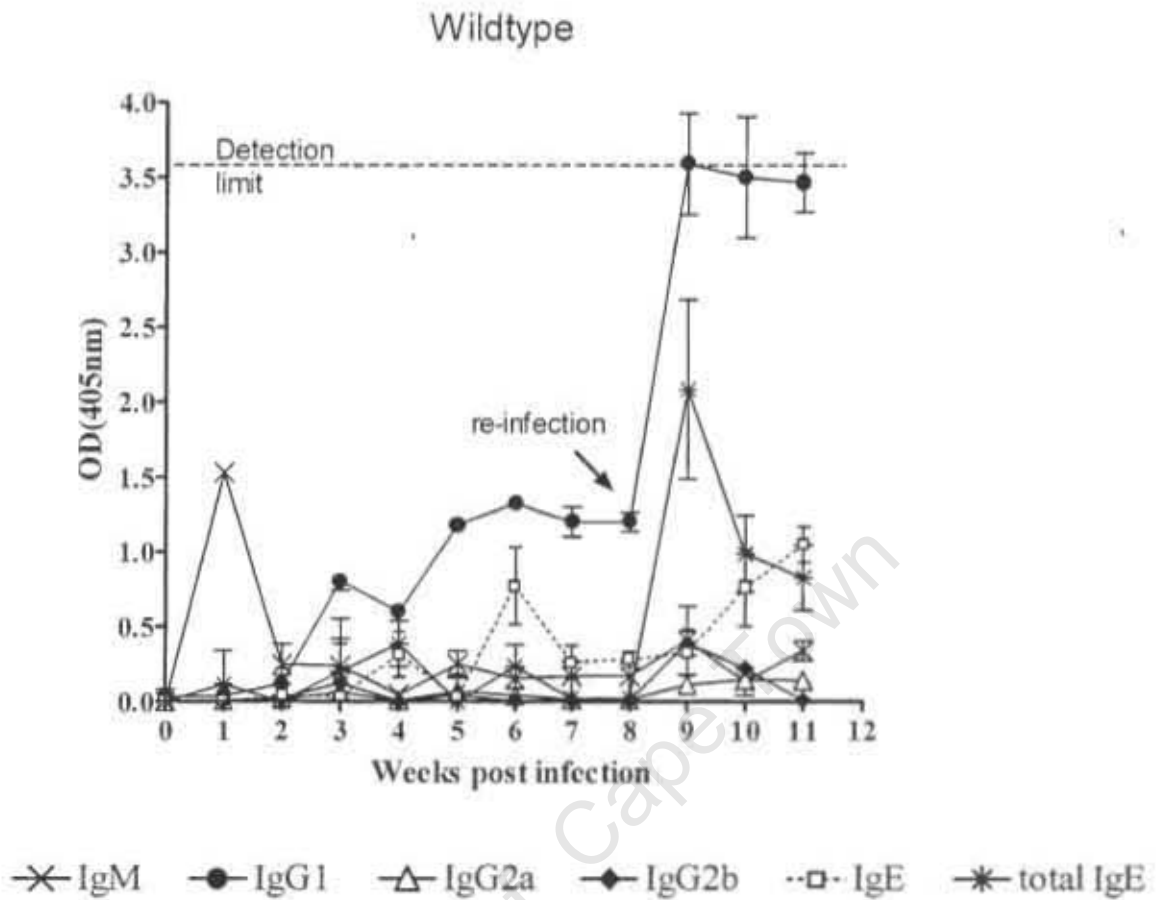


Figure 2.4.14. Wildtype mice exhibit strong B-cell memory responses to *Anisakis*. Wildtype mice ($n = 4$) were infected with 2 *Anisakis* larvae at week 0 and re-infected at week 8. Antibody responses were measured by ELISA. Results are representative of 2-3 experiments. Data presented as mean \pm SD.

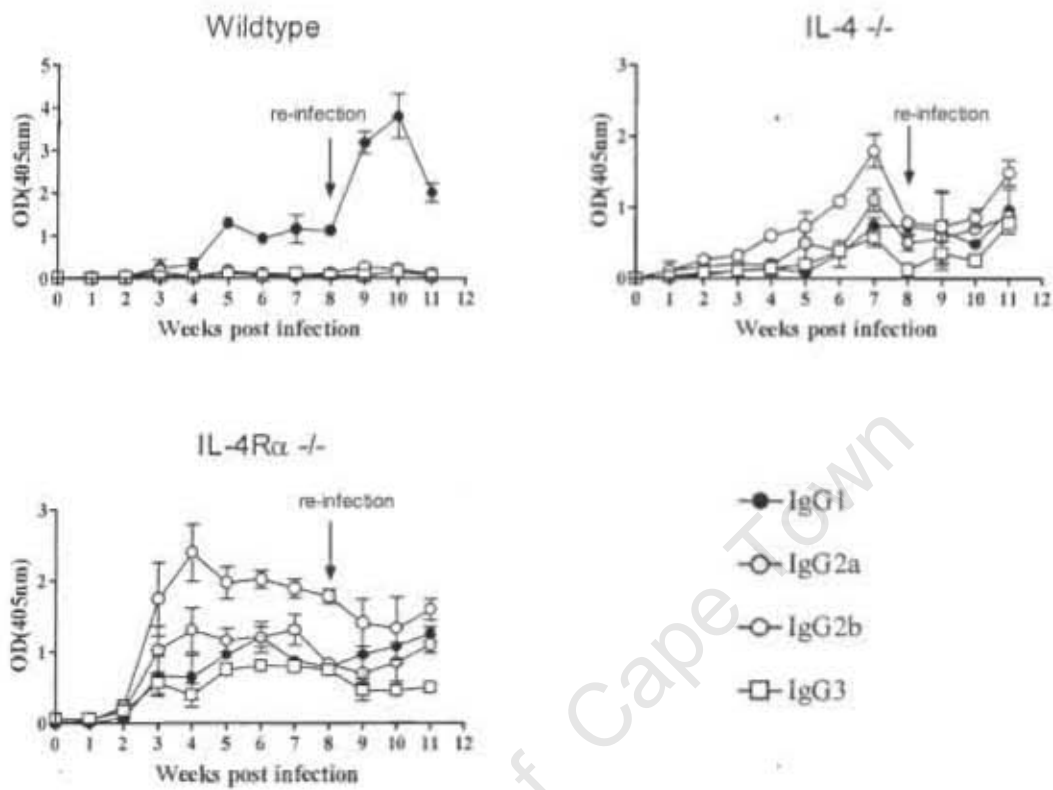


Figure 2.4.15. IgG isotype production after re-infection. Mice were infected with 2 *Anisakis* larvae at week 0 and re-infected at week 8. Antibody responses were measured by ELISA. Results are representative of 2-3 experiments (n = 3-5). Data presented as mean \pm SD.

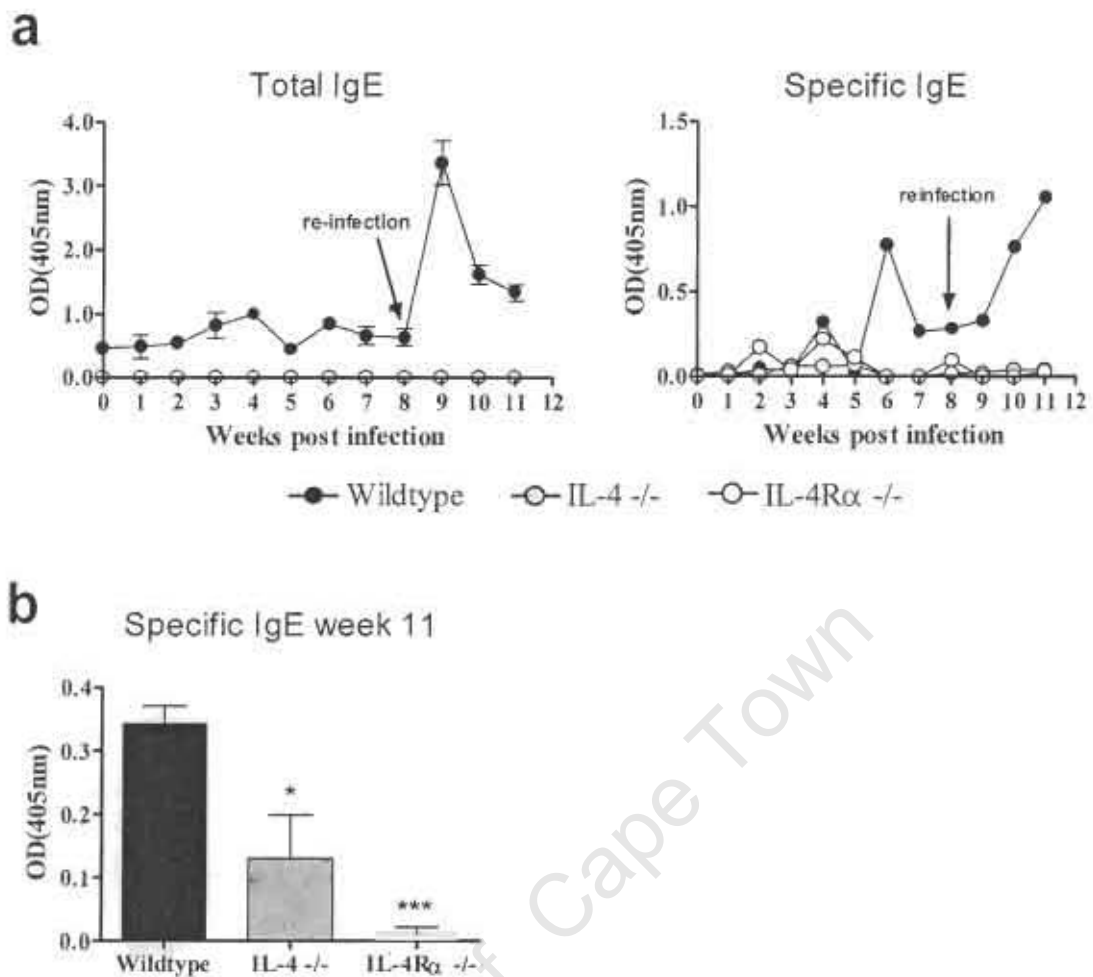


Figure 2.4.16. IgE production after re-infection. Mice were infected with 2 *Anisakis* larvae at week 0 and re-infected at week 8. **a)** Antibody responses in individual (total IgE) or pooled (specific IgE) sera were measured by ELISA. Results are representative of 2-3 experiments (n = 3-5). Data presented as mean +/- SD. **b)** In one experiment IL-4 -/- mice produced low levels of specific IgE (but not total IgE)(n = 4). Data presented as mean +/- SEM. Statistical significance between wildtype and knockout mice is shown. *, P<0.05; ***, P<0.001.

2.4.8 Antibody responses after oral re-exposure to *Anisakis* proteins

Patients may re-encounter *Anisakis* proteins in the flesh of fish after they have had live infections with *Anisakis* larvae, therefore previously infected mice were boosted orally with a small dose (500 μ g) of *Anisakis* extract and subsequent antibody responses were examined. Interestingly, the most noticeable change in antibody levels was a strong increase in IgA in IL-4R α $-/-$ mice (Fig. 2.4.17). This did not occur when mice were re-infected with live larvae (Fig. 2.4.18). There was also a slight trend towards decreased IgG2b and IgG3 in IL-4R α $-/-$ mice. Wildtype mice did not show the same dramatic increase in IgG1 levels as they did after intraperitoneal re-infection with live larvae. However, there may have been a slight increase in IgG2a and IgG2b levels following the oral boost.

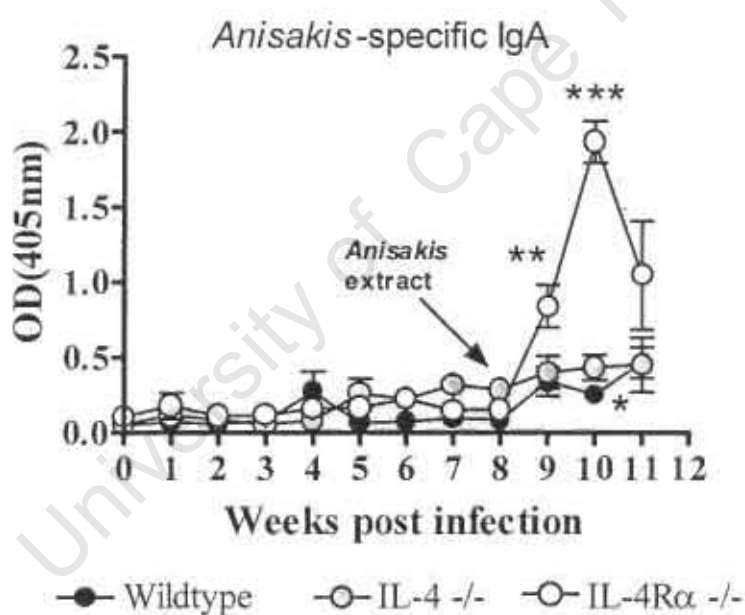


Figure 2.4.17. *Anisakis*-specific IgA rises significantly in IL-4R α $-/-$ mice after an oral boost with *Anisakis* extract. Mice (n = 4-5) were infected with 2 *Anisakis* larvae at week 0, and boosted orally with 500 μ g of *Anisakis* extract at week 8. Antibody responses were measured by ELISA. Results are representative of 2 experiments. Data presented as mean \pm SD. Statistical significance between week 0 and other timepoints is shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

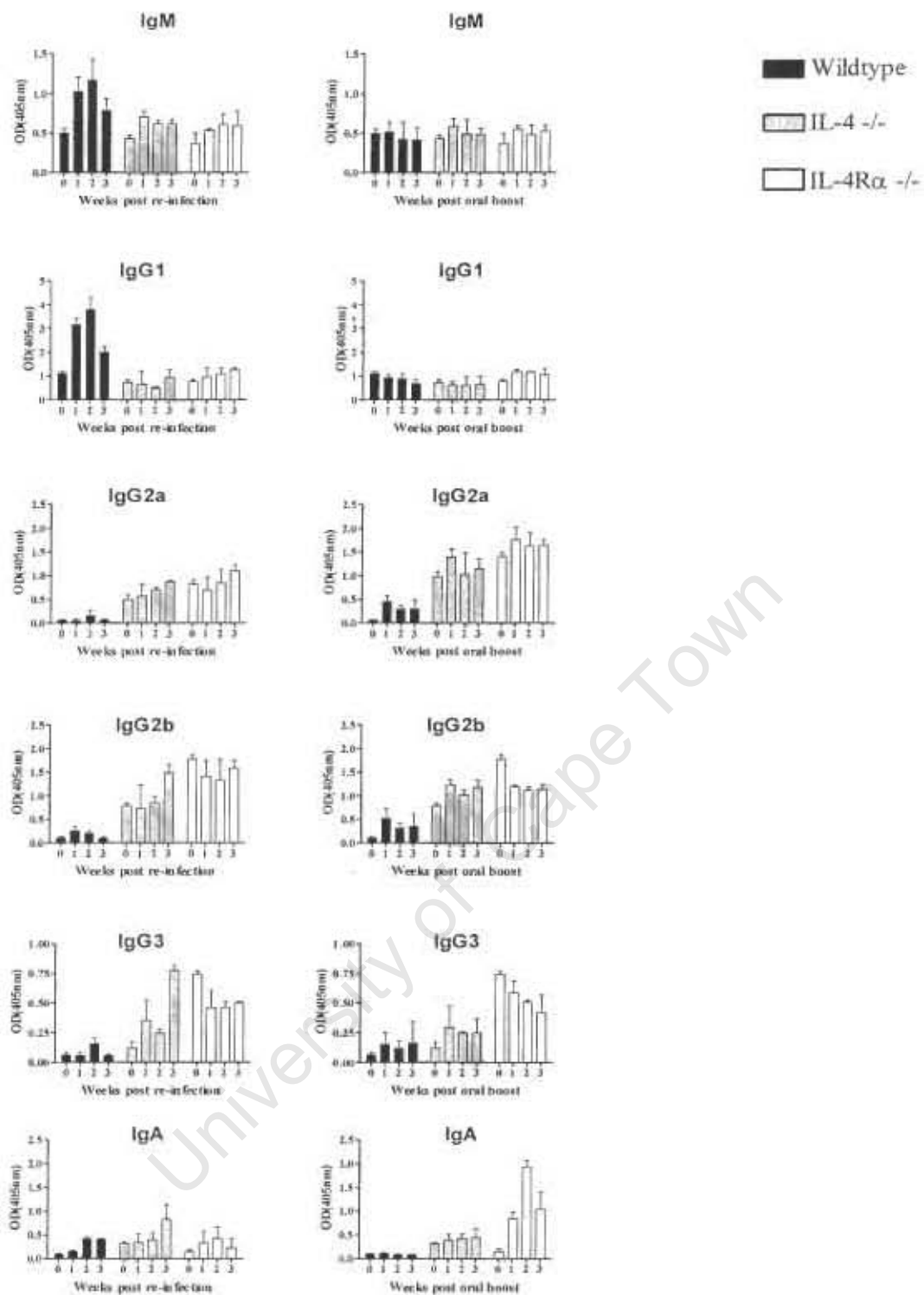


Figure 2.4.18. Comparison between *Anisakis*-specific antibody responses after re-infection with live larvae and oral boost with *Anisakis* extract. Results are representative of 2 experiments ($n = 3-5$). Data presented as mean \pm SEM.

2.4.9 Allergic reactions due to ingestion of *Anisakis* proteins

It is controversial whether live *Anisakis* is needed in order to produce allergic reactions, or whether ingestion of proteins alone can cause reactions (Audicana 2002). To investigate whether *Anisakis* proteins can cause allergic reactions, wildtype mice were infected with *Anisakis* larvae (wk 0), re-infected to boost responses (wk 8), and then orally challenged with 5mg of *Anisakis* extract (wk 11)(Fig.2.4.19). Sham-infected mice challenged with *Anisakis* extract were used as a control for the effects of *Anisakis* extract administration by oral gavage.

At week 11, levels of type 2 antibodies (IgE, IgG1) were high (Fig.2.4.20a) and Th2 type cytokines were present (Fig. 2.4.20b). Ingestion of *Anisakis* proteins by previously sensitized mice induced mild allergic reactions within an hour, including scratching, irritability, reduced activity, diarrhoea and puffiness around eyes (Fig. 2.4.21a). Levels of serum mouse mast cell protease (MMCP)-1, a marker of mast cell degranulation, and plasma histamine, a vasoactive mediator, were increased after re-infection and further boosted after oral challenge (Fig. 2.4.21b). Lung histopathological analysis showed that ingestion induced mucus hypersecretion by goblet cells and cellular infiltration, both common signs of airway hypersensitivity (Fig.2.4.22a). Levels of MMCP-1 were also increased in lung homogenate samples of the experimental group as compared to sham-infected controls, while EPO and eotaxin levels were not increased. These results demonstrate that infection with *Anisakis* can predispose to *Anisakis* allergy, and that proteins of the dead larvae alone are sufficient to cause allergic reactions in sensitized mice.

2.4.10 IL-4, IL-13 and IL-4R α are important in *Anisakis*-induced hypersensitivity

Infection with *Anisakis* followed by oral challenge with *Anisakis* extract resulted in visibly reduced signs of allergy in IL-4 $-/-$ mice compared to wildtype controls (Fig. 2.4.23a) Irritability was present, but diarrhoea occurred less frequently than in wildtype mice and was less severe. Activity was normal. In line with this, serum MMCP-1 and plasma histamine levels were impaired (2.4.23b,c). Furthermore, while lung goblet

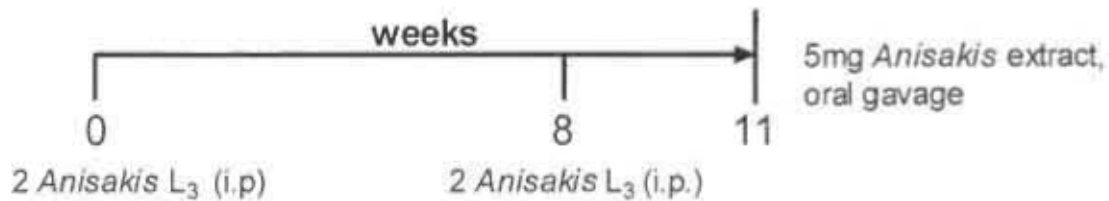


Figure 2.4.19. Protocol for challenge experiments. Mice were infected with 2 *Anisakis* larvae at week 0 and re-infected with the same at week 8. 5mg of *Anisakis* extract was used to challenge mice by oral gavage at week 11.

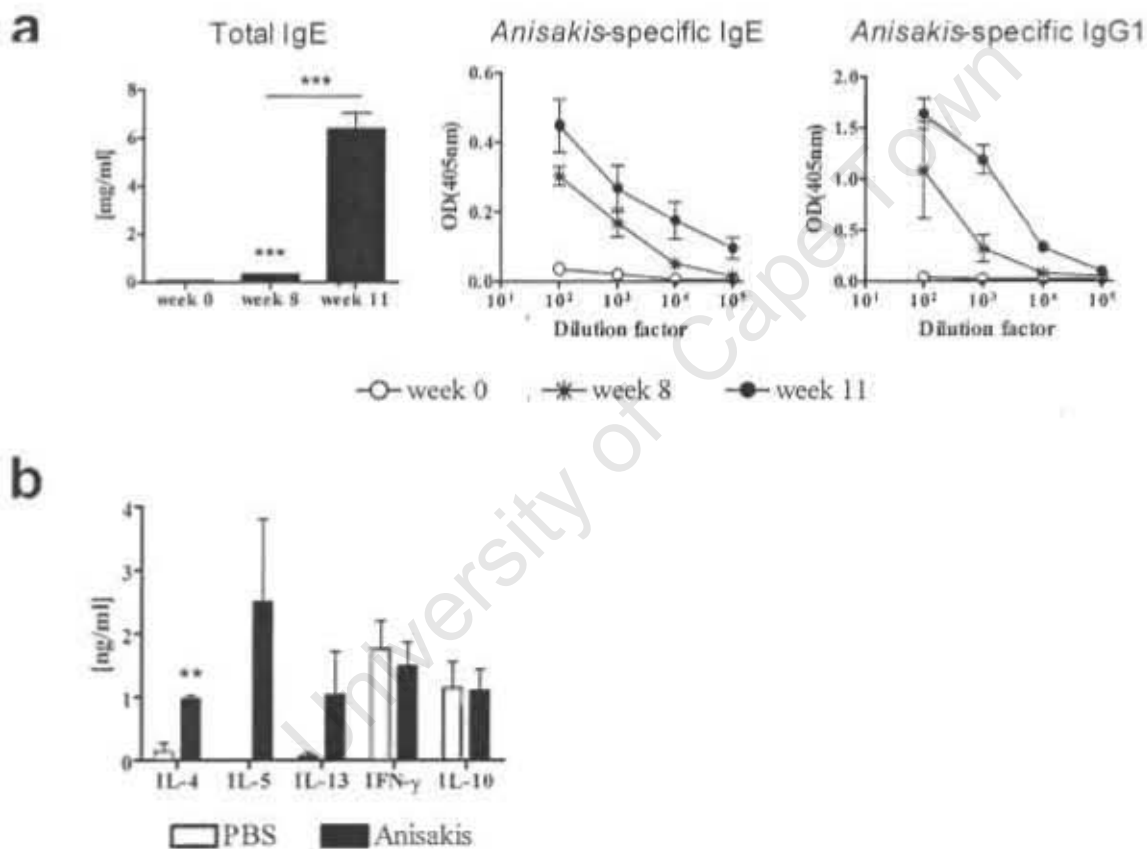


Figure 2.4.20. Antibody and cytokine responses on the day of challenge. **a**, Levels of type 2 antibodies are high at the time of challenge. **b**, Splenocytes from individual spleens (n=5) restimulated with anti-CD3 tend towards a predominant Th2 profile. Data presented as mean +/- SEM. Statistical significance between PBS- and *Anisakis* sensitized mice is shown **, P<0.01; ***, P<0.001.

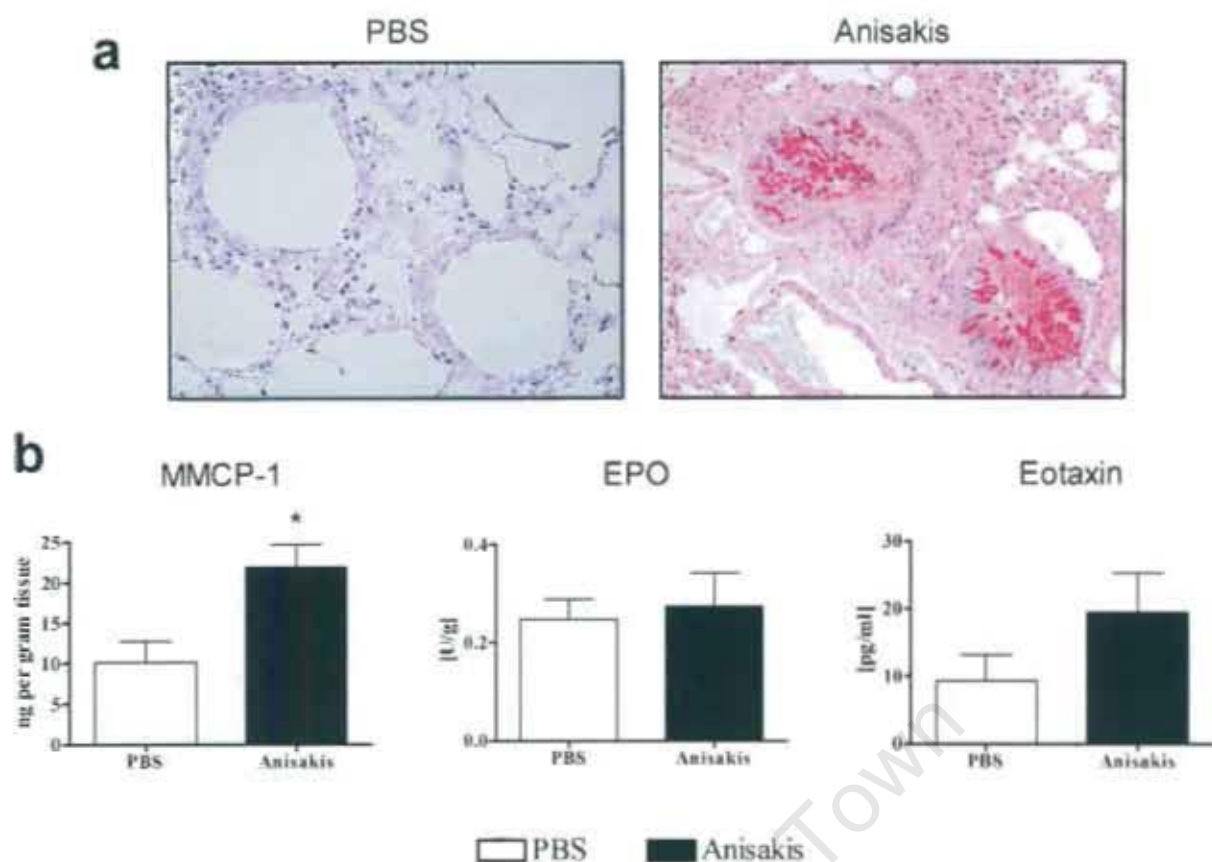


Figure 2.4.22. Lung pathology after *Anisakis* challenge in wildtype mice. Mice were infected with 2 *Anisakis* larvae or sham-infected with PBS at week 0, and challenged by oral gavage with *Anisakis* extract at week 11. **a**, Lungs were stained with PAS for mucus production. Goblet cell hyperplasia, mucus hypersecretion and inflammatory infiltrate is seen in *Anisakis*-sensitized and challenged mice. **b**, MMCP-1, EPO and eotaxin levels were measured in lung homogenates from challenged mice (n = 4). Data presented as mean +/- SEM. Statistical significance between PBS- and *Anisakis*-sensitized mice is shown *, P<0.05.

cell hyperplasia and mucus secretion sometimes occurred it was both less prevalent and less pronounced than in wildtype mice (Fig. 2.4.24a). The pictured IL-4 $-/-$ lung shows the highest degree of mucus hypersecretion ever seen in this strain. In some mice, mucus hypersecretion was scanty or absent. Furthermore, while many eosinophils and neutrophils were seen in bronchiolar lavage fluid from wildtype mice, the infiltrate in IL-4 $-/-$ mice consisted primarily of macrophages and lymphocytes (Fig.2.4.24b,c)

In IL-4R α $-/-$ mice, allergic reactions were completely abolished and serum MMCP-1 and plasma histamine levels were impaired (Fig.2.4.23). Mucus was never present in the lungs, which showed clear airways comparable to those found in sham-infected controls (Fig.2.4.24a). Only small numbers of cells were found in bronchiolar lavage fluid (Fig. 2.4.24b,c). These cells appeared to be resident alveolar macrophages.

Local antibody production was examined in lung homogenates, and skewing towards type 1 antibodies was found in IL-4 $-/-$ and IL-4R α $-/-$ mice, with reduced and impaired IgG1 respectively, but increased IgG2a (Fig.2.4.25). Total IgE production was impaired in the lungs of both knockout strains. At the week of challenge, systemic antibody production was strongly skewed towards type 2 antibodies in wildtype mice, while knockout strains produced high levels of IgG2a and IgG2b in addition to IgG1 (Fig.2.4.26a). Total IgE was extremely low in the knockout strains, and only low levels of specific IgE were detected in IL-4 $-/-$ mice (Fig.2.4.26b).

Cytokine production by splenocytes restimulated with anti-CD3 was examined at week 11 (Fig.2.4.26c). Splenocytes from wildtype mice showed a stronger skewing towards Th2 cytokine production than either knockout. IL-4 and IL-13 were produced by IL-4R α $-/-$ mice, though this strain would not be expected to respond to these cytokines. Splenocytes from IL-4 $-/-$ mice produced decreased amounts of IL-13. IL-5 production was similar in all strains, while there was a trend towards decreased IL-9 and increased IFN- γ production in the knockout strains.

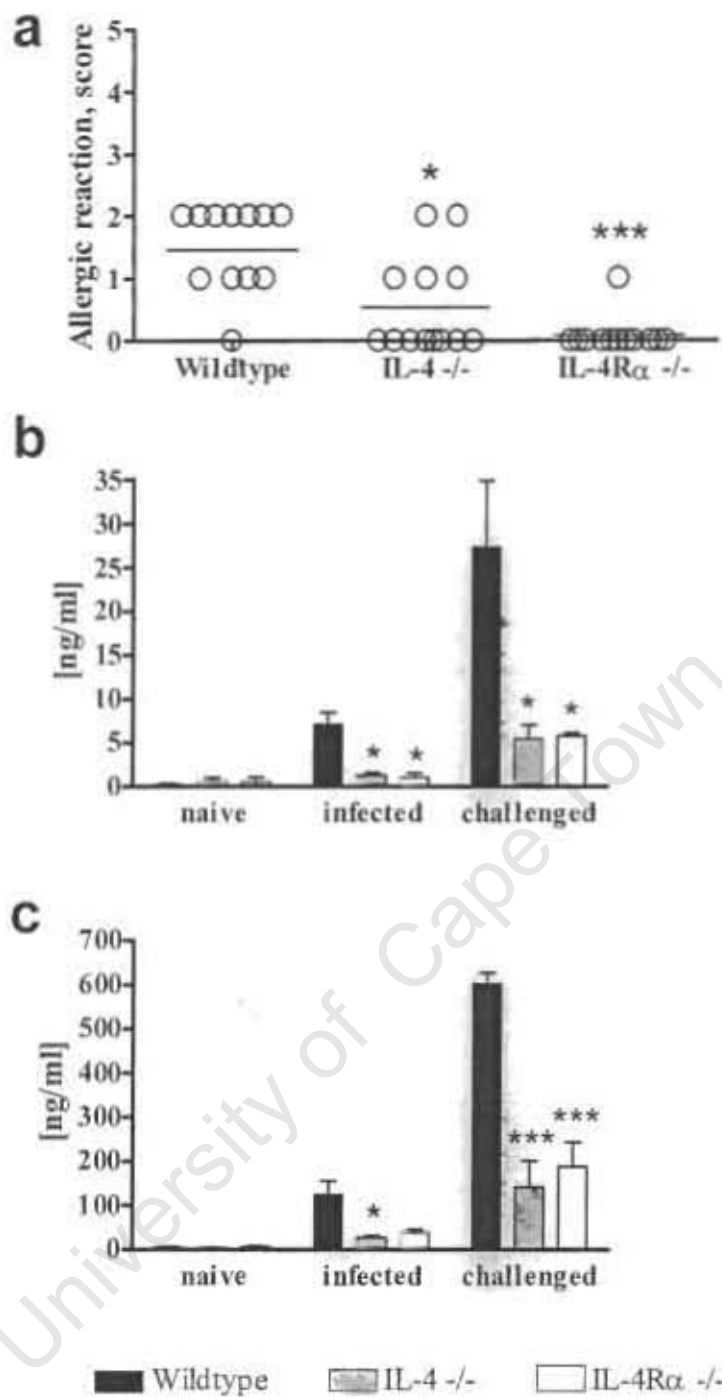


Figure 2.4.23. Allergic reactions were reduced in IL-4^{-/-} and IL-4Rα^{-/-} mice. Mice were infected with 2 *Anisakis* larvae (week 0), re-infected with the same (week 8) and challenged orally with *Anisakis* extract (week 11). **a**, Allergic reactions were scored (see Methods). Open circles represent individual mice (pooled from 3 experiments, n = 4-5). Data presented as mean +/- SEM. **b**, Plasma histamine was reduced in IL-4^{-/-} and IL-4Rα^{-/-} mice. **c**, Serum MMCP-1 levels were reduced in IL-4^{-/-} and IL-4Rα^{-/-} mice. Data presented as mean +/- SEM. Statistical significance between wildtype and knockout mice is shown. *, P<0.05; **, P<0.01; ***, P<0.001.

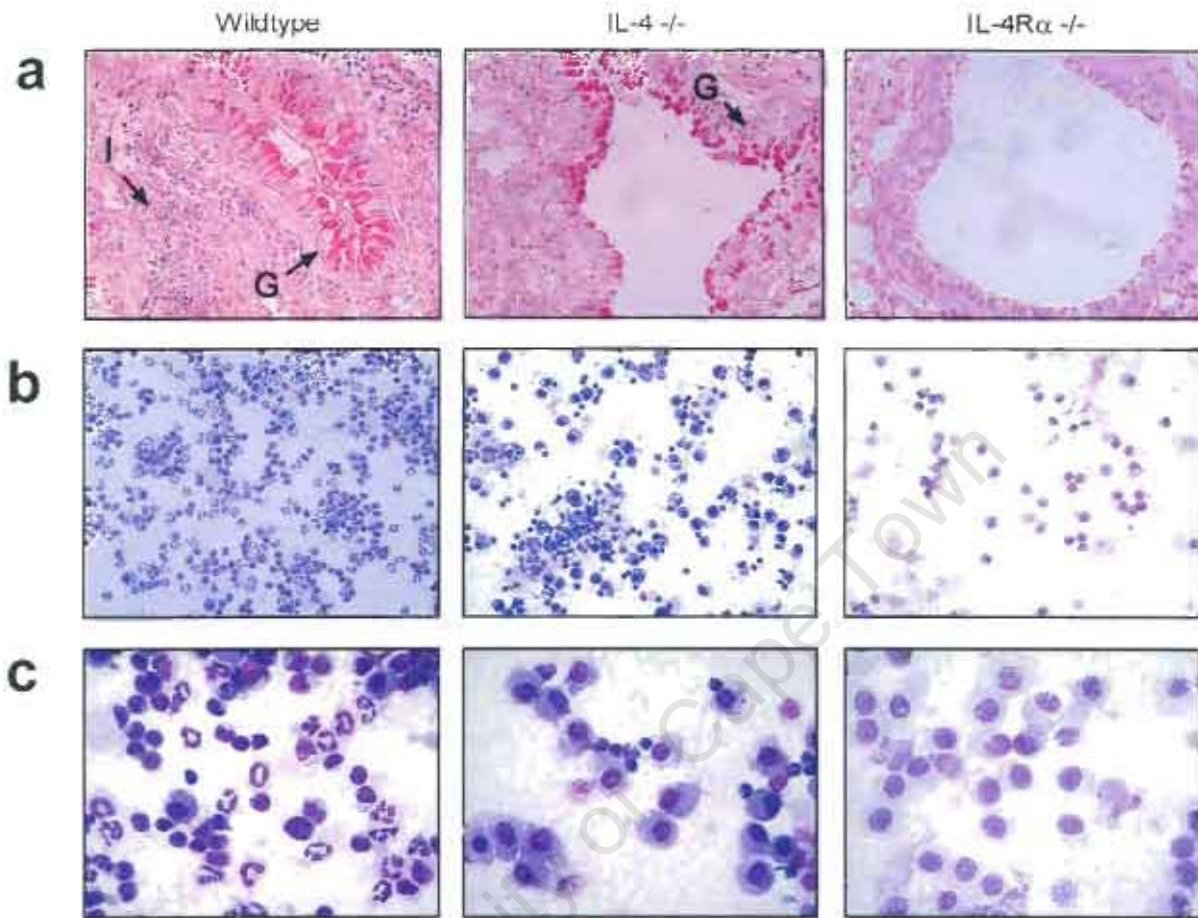


Figure 2.4.24. Lung pathology is reduced and abrogated in IL-4^{-/-} and IL-4Rα^{-/-} mice respectively. Mice were infected with 2 *Anisakis* larvae at week 0, re-infected at week 8 and challenged orally with 5mg *Anisakis* extract. **a**, Lungs were stained with PAS for mucus. **b**, Bronchiolar lavage cells (200x). **c**, Bronchiolar lavage cells (1000x).

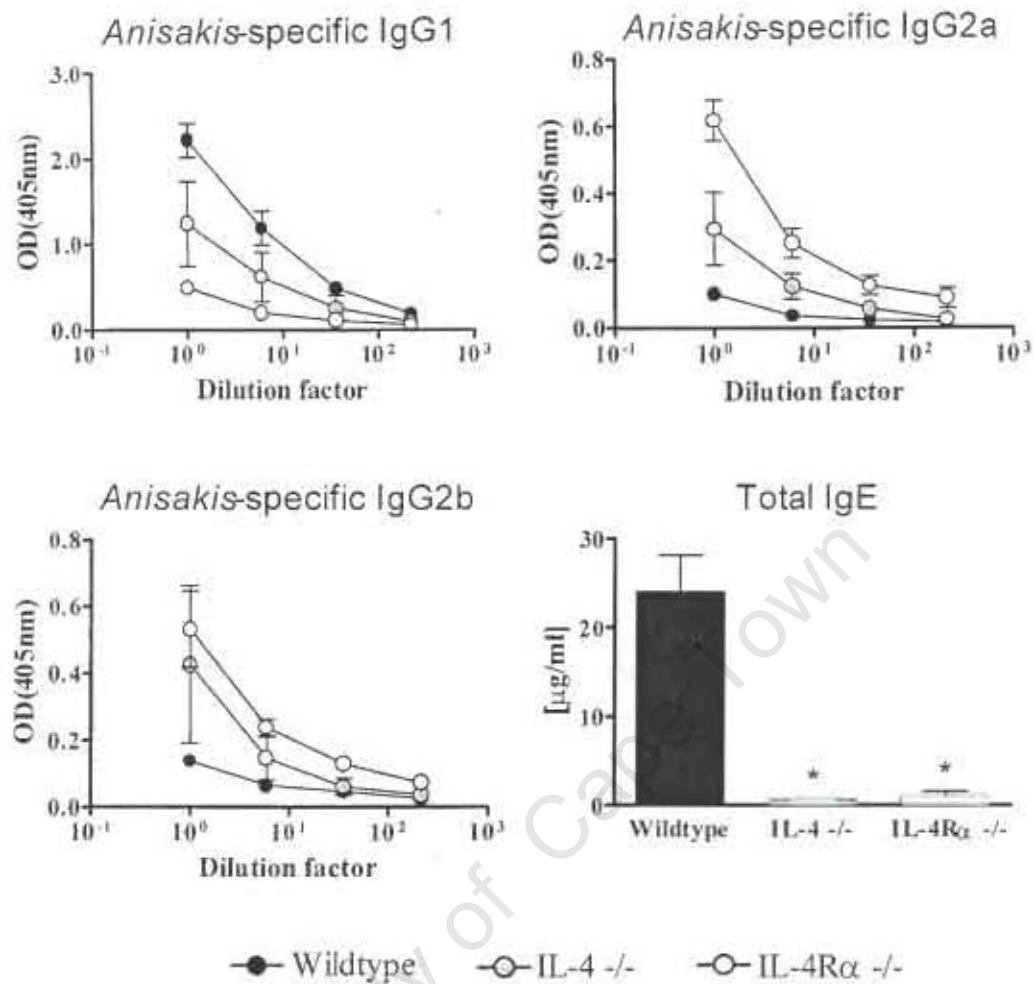


Figure 2.4.25. Type 2 antibodies were reduced in lung homogenates of IL-4 and IL-4R α knockouts. Lungs were homogenized in PBS and antibodies were measured by ELISA. Data presented as mean \pm SEM. Statistical significance between wildtype and knockout mice is shown. *, P<0.05.

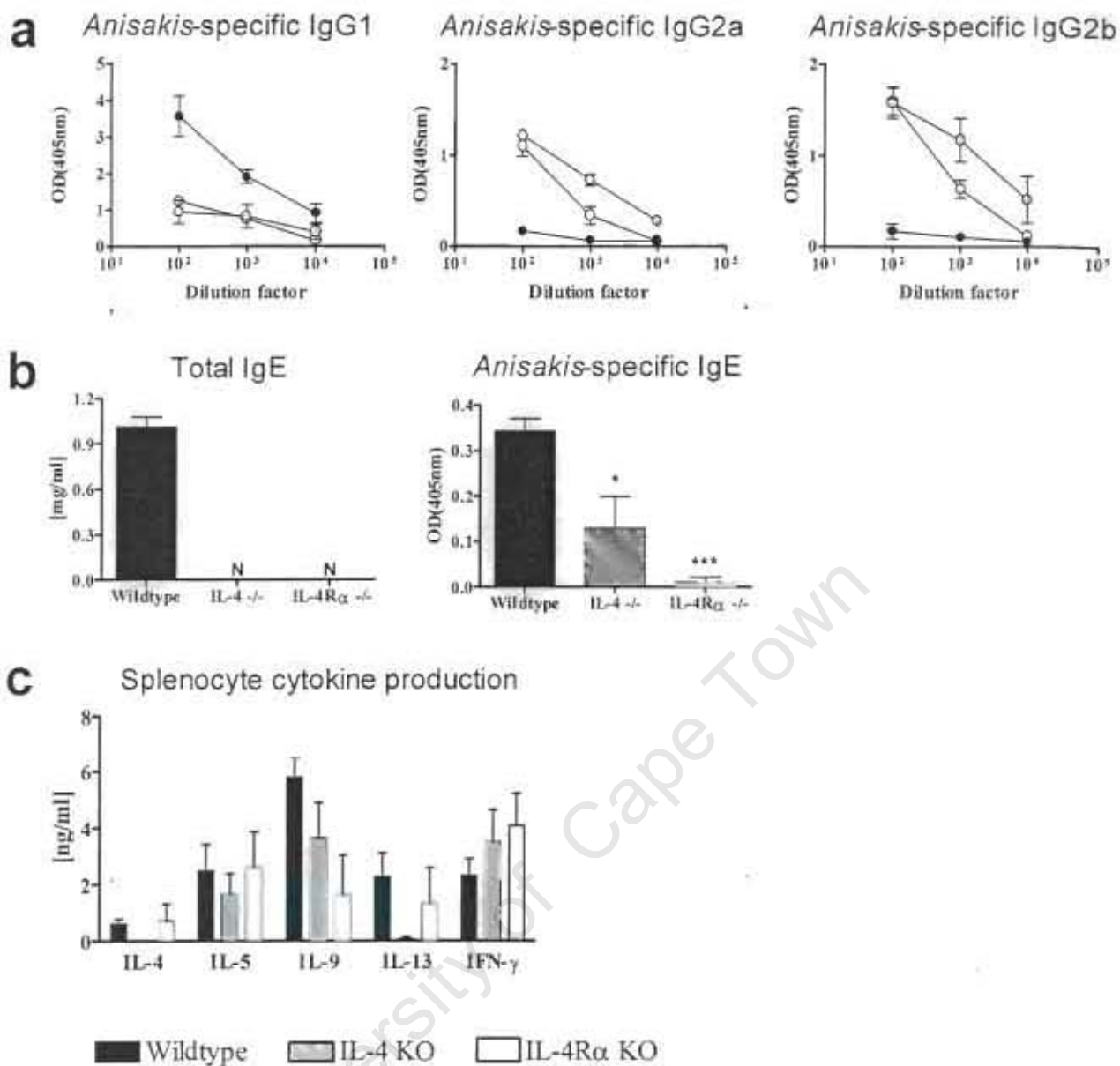


Figure 2.4.26. Systemic antibodies and cytokine production in wildtype, IL-4^{-/-} and IL-4Rα^{-/-} mice at week 11 post infection. Serum IgG (a) and IgE (b) antibodies were measured by ELISA (n = 4-5). c) Cytokine production by splenocytes (n = 4-5) restimulated with anti-CD3 was measured by ELISA. Data presented as mean +/- SEM. Statistical significance between wildtype and knockout mice is shown. N, not detectable; *, P<0.05; **, P<0.01; ***, P<0.001.

2.4.11 Oral sensitization with *Anisakis* extract

Weekly doses of 1mg *Anisakis* extract or PBS were administered to wildtype BALB/c mice by oral gavage for 5 weeks. On the 6th week mice were challenged orally with 5mg *Anisakis* extract or intravenously with 750µg of *Anisakis* extract. Neither oral nor intravenous challenged mice appeared to have allergic reactions.

By the time of challenge (week 6), serum levels of *Anisakis*-specific IgG1, IgG2a, IgG2b and total and specific IgE were raised in *Anisakis*-dosed mice compared to PBS dosed controls (Fig. 2.4.27). This is different to the isotype profile seen after live *Anisakis* infection, in which IgG2a and IgG2b remain low in wildtype mice (Fig. 2.4.13). However, in both cases IgG1 is the predominant IgG isotype produced.

Splenocytes from *Anisakis*-dosed mice restimulated with conA or anti-CD3 did not show any significant differences in production of IL-4, IL-5, IL-10 or IL-13 compared to PBS-dosed controls (Fig. 2.4.28). However, IFN-γ production was significantly reduced in *Anisakis*-dosed mice. Other oral sensitization protocols tested (data not shown) also did not show any significant differences between Th2 type cytokine production of splenocytes from *Anisakis*- and PBS-dosed mice. This is unlike the strong Th2 cytokine production stimulated by live *Anisakis* larvae.

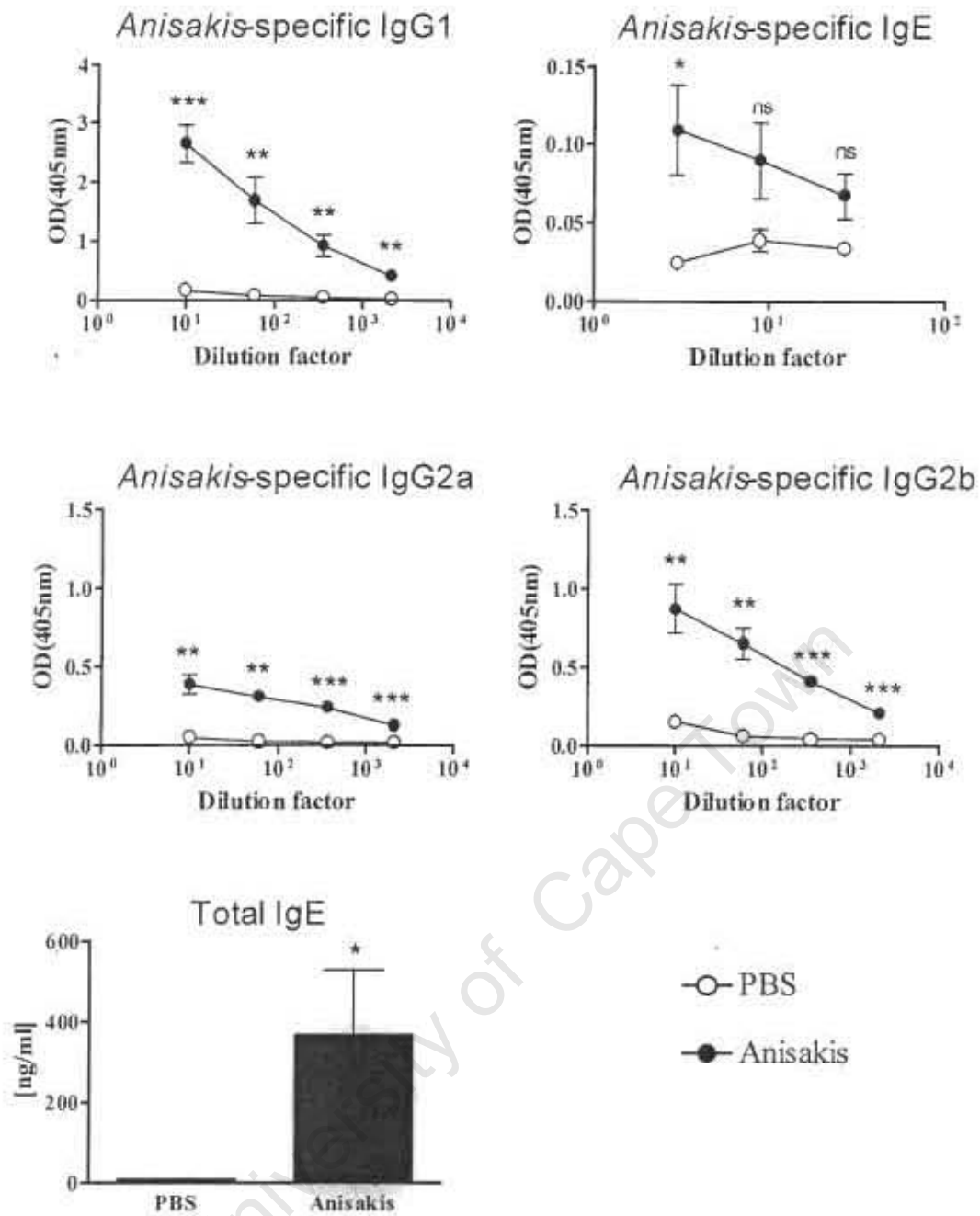


Figure 2.4.27. Antibodies induced by oral sensitization with *Anisakis* extract. Weekly doses of 1mg of *Anisakis* extract was administered to wildtype mice by oral gavage for 5 weeks. Serum antibodies were measured at week 6 (n = 4) Data presented as mean +/- SEM. Statistical significance between PBS- and *Anisakis*-sensitized mice is shown. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.

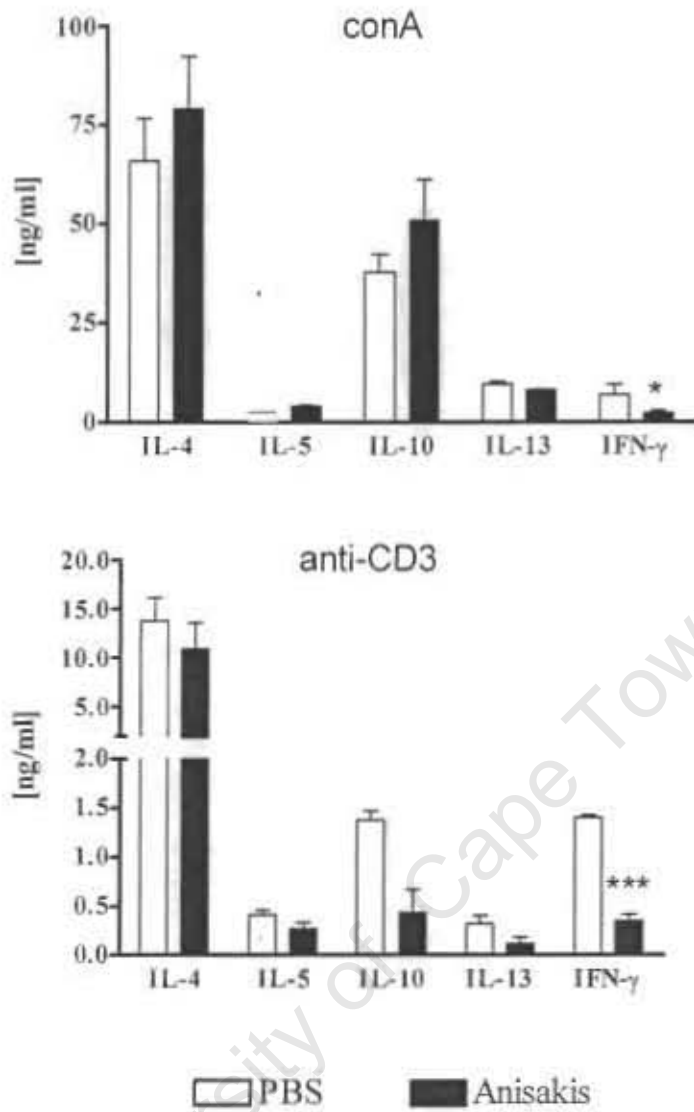


Figure 2.4.28. IFN- γ production by resimulated splenocytes was decreased in mice sensitized orally with *Anisakis* extract. Splenocytes (pooled, n = 4) were plated in triplicate and restimulated with conA or anti-CD3 and supernatant cytokines were measured by ELISA. Data presented as mean \pm SEM. Statistical significance between PBS- and *Anisakis*-sensitized mice is shown. *, P<0.05; **, P<0.01; ***, P<0.001.

2.5 DISCUSSION

In this study, a murine model was used to gain a better understanding of the underlying immunological mechanisms of *Anisakis* infection and related allergic responses. The results show for the first time that infection with *Anisakis* induces strong, systemic Th2 responses which can sensitize mice to react to subsequent oral challenge with *Anisakis* proteins, and that IL-4, IL-13 and the IL-4R α are critical in this process.

Anisakis infection was performed by intraperitoneal injection, as in previous studies^{88, 89}. Immune responses of wildtype BALB/c mice were compared with those of mice deficient in IL-4, IL-13 and the IL-4R α subunit, required for signaling of both IL-4 and IL-13, in order to determine the importance of these cytokines in *Anisakis*-related disease. *Anisakis* infection was transient in all mouse strains, with the death of the larvae occurring within approximately 3 weeks, which corresponds to previous findings^{88, 89}. There was no obvious difference in the time of larval survival between mouse strains, but as only a few larvae could be located, no conclusions are possible concerning the role of IL-4, IL-13 and IL-4R α on larval survival.

In all strains except IL-13^{-/-} mice, peripheral eosinophilia rose after *Anisakis* infection with peaks of varying intensity around 3 to 4 weeks post infection, which is in correlation with the approximate time of larval death. Conversely, a previous murine model found a slight drop in peripheral blood eosinophils in the early stages of infection, but this study was performed with CBA/J mice⁸⁸. Peripheral blood eosinophilia in human anisakiasis is similarly variable^{35, 37}. Infiltration of eosinophils to the area of the larval invasion is thought to be more important, with eosinophil products such as eosinophilic cationic protein, major basic protein, nitric oxide and peroxidases thought to be primarily responsible for the destruction of the larvae^{42, 72, 83}. In all strains of mice, peritoneal lavages taken after infection showed increased numbers of eosinophils starting as early as one day post infection. Experiments have shown *Anisakis* extract to be chemotactic for eosinophils^{81, 82}. Neutrophils were also found infiltrating the peritoneal cavity, as early as day 1 post infection. At a later stage, approximately 2 weeks post infection, numbers of

infiltrating macrophages and lymphocytes were raised. IL-4R α $-/-$ and IL-13 $-/-$ mice had particularly high numbers of infiltrating cells, and displayed enlarged spleens.

In wildtype mice, *Anisakis* infection induced Th2-biased immune responses with elevated synthesis of IgE and IgG1, little IgG2a or IgG2b, and increased splenocyte production of IL-4, IL-5, IL-9 and IL-13. In contrast, IL-4 $-/-$ and IL-4R α $-/-$ mice had elevated levels of IgG2a, IgG2b and IgG1, barely detectable IgE levels, and reduced Th2 type cytokine production compared to wildtypes. Re-infection of wildtype mice with live larvae resulted in strong memory responses leading to vast increases in IgG1 and IgE, and susceptibility to food-allergic type reactions following oral administration of *Anisakis* extract. Histological examination of lung tissue demonstrated that the airways of orally challenged mice were obstructed by inflammatory cells and displayed profound goblet cell hyperplasia and mucus. Respiratory symptoms, including mucus hypersecretion, are a common feature of food allergy and increase the risk of fatality, although the mechanism by which ingestion of allergens induces lung pathology is unknown¹⁰⁰⁻¹⁰³. The recruitment of Th2 effector cells to the airway has been associated with eosinophilia and mucus hypersecretion¹⁰⁴. Several of the Th2 cytokines induced by *Anisakis* infection (IL-4, IL-5, IL-9, IL-13) play important roles in airway hyperreactivity^{105, 106}. This may explain cases of asthma thought to be related to consumption of *Anisakis*-contaminated fish¹³. However, the airway pathology after oral ingestion of *Anisakis* proteins in this model is stronger than might be expected, raising the possibility that minute amounts of *Anisakis* antigen may have directly reached the airways by aspiration. If the pathology is due to inhalation, rather than ingestion of allergens, this would indicate that *Anisakis* proteins additionally have the ability to cause asthma if inhaled by pre-sensitized mice. Recent studies performed in our laboratory confirm this ability (Kirsten et al., unpublished observations). Furthermore, in certain protocols, mice infected with *Anisakis* exhibit some mucus hypersecretion in the lungs even prior to challenge with *Anisakis* extract (Kirsten et al., unpublished observations). This may be due to high levels of Th2 cytokine production.

Sensitization by oral administration of *Anisakis* extract alone induced *Anisakis*-specific antibody production but did not lead to the striking increase in Th2 cytokine production elicited by live infection. Furthermore, orally sensitized mice did not react to an oral challenge despite the presence of IgE antibodies, suggesting tolerance. This may indicate that live infection with *Anisakis pegreffii* bypasses oral tolerance, inducing strong systemic Type 2/Th2-like responses that predispose mice to develop allergic reactions upon subsequent ingestion of *Anisakis* proteins. Taking into consideration that the sensitization was performed in the absence of an adjuvant (to better reflect a typical human situation), the observed allergic reactions in mice after sensitization with live larvae were strong for a food-borne allergen. Most animal models for food allergies require adjuvants such as cholera toxin or alum in order to trigger allergic reactions^{99, 107}. A recent study sensitized mice intraperitoneally with a combination of *Anisakis simplex* extract, pertussis toxin and alum¹⁰⁸. These mice reacted to an intravenous challenge with anaphylaxis, but showed no reactions after oral challenge.

It has long been debated whether infection with live *Anisakis* larvae is required for sensitization and/or allergic reactions^{5, 109}. This study demonstrates that whilst infection certainly produces the potent Th2/type 2 response which is likely to be responsible for sensitization to anaphylaxis, exposure to *Anisakis* proteins alone may be enough to elicit allergic reactions in strongly sensitized individuals. Whether or not allergic reactions occur in response to *Anisakis* proteins probably depends upon the balance between exposure to live larvae and larval proteins alone, as well as on the genetic disposition of each individual. Frequent ingestion of *Anisakis*-contaminated fish may boost antibody and cytokine responses, contributing to chronic urticaria.

In contrast to wildtype mice, IL-4^{-/-} and IL-4R α ^{-/-} mice did not demonstrate strong increases in antibody production after re-infection. Furthermore, allergic reactions were reduced and absent in IL-4 and IL-4R α ^{-/-} mice respectively. In IL-4^{-/-} mice, lung mucus hypersecretion was decreased, and inflammatory infiltrate into the lungs was composed primarily of lymphocytes and macrophages. This probably reflects the importance of IL-4 in the development of Th2 cells producing Th2 effector cytokines

such as IL-5, IL-9 and IL-13. Production of these cytokines was diminished in this strain. Some allergic reactions such as diarrhoea did occur (at a reduced prevalence) in IL-4 $-/-$ mice, probably due to IL-13 mediated responses, since IL-4R α $-/-$ mice, which lack both IL-4 and IL-13 signaling, did not show reactions. Furthermore, in IL-4R α $-/-$ mice lung pathology was entirely abrogated. This supports recent findings that IL-13 plays a key role in mucus hypersecretion in mice ¹¹⁰⁻¹¹². In humans, the role of IL-13 may be even more significant because unlike mouse B cells, human B cells possess a functional IL-13 receptor ¹¹³. These data indicate that the IL-4R α may play as crucial a role in food allergy as it does in allergic asthma, with important therapeutic implications.

The results of this study show that wildtype and IL-4R α $-/-$ mice have polar immune responses to *Anisakis*. Wildtype mice show the least inflammation as a result of *Anisakis* infection but exhibit the strongest memory responses after re-infection and demonstrate allergic reactions following oral challenge with *Anisakis* extract. In contrast, IL-4R α $-/-$ mice show the strongest inflammatory response to *Anisakis* infection, but little memory response upon re-infection, and do not react after oral challenge. Extrapolating these results to humans may suggest that prior infections are more likely to be asymptomatic in individuals experiencing allergic reactions to *Anisakis*. This is in line with the fact that individuals experiencing allergic reactions to *Anisakis* for the first time typically do not recall prior episodes of gastric or intestinal anisakiasis, despite the fact that they already have *Anisakis*-specific IgE, and so must have had previous exposures ^{43, 53}. Furthermore, gastrointestinal pain is normally far less severe in these patients than it is in patients without allergic reactions, and may even be absent. Interestingly, gastroallergic anisakiasis patients often have no prior history of allergy ¹¹⁴, but *Anisakis* is unusual in that it is both an etiological agent of allergies and a parasitic helminth.

The excretory-secretory products of helminths such as *Anisakis* spp. are particularly good at eliciting Th2 responses ¹¹⁵. Parasitic helminths release large amounts of proteases which facilitate the penetration of host tissues and the digestion of tissues for nutrients ^{116, 117}. Proteases are therefore amongst the first parasitic antigens to which the host is exposed, and a growing body of data on allergens indicates that proteolytic activity may

be a key biochemical property in conferring upon a protein the ability to elicit IgE and Th2 cytokine responses. Important allergens which are proteases include Der p 1, Fel d 1 and Bla g 2, the major allergens of house dust mite, cat and cockroach respectively, as well as major allergens of moulds and fruits, and the strongly allergenic bacterial enzymes found in detergents (reviewed by Donnelly et al.)¹¹⁶. Protease allergens such as Der p 1 have the ability to cleave proteins that make up the tight junctions between epithelial cells lining the lungs, nasal passages and gut. In this way they bypass the physical barriers of the body and gain abnormal access to subepithelial immune cells. Access to subepithelial immune tissue rich in antigen-presenting cells is considered a primary risk factor for the development of allergic sensitization. The secretion of proteolytic enzymes may therefore explain how *Anisakis* larvae and proteins bypass the tolerance mechanisms associated with responses to most ingested substances. Proteolytic activity by allergens can also directly influence immune responses by cleaving various receptors and costimulatory molecules on dendritic cells, T cells and mast cells (reviewed by Donnelly 2006)¹¹⁶. Experimental data demonstrates that concomitant administration of Der p 1 or mould proteases with normally tolerogenic antigen renders it allergenic. Therefore the allergenicity of *Anisakis* extract may be due to its protease content. Other parasites such as *Nippostrongylus brasiliensis*, *Ascaris lumbricoides* and *Toxocara canis* also exhibit allergenicity¹¹⁸⁻¹²⁰.

Despite the fact that parasites are particularly good at eliciting Th2 responses¹²¹, many studies have paradoxically indicated that parasite infection may protect against allergic disease¹²²⁻¹²⁴. Experimental mouse models indicate that this protection is mediated by IL-10 and/or regulatory T cells^{125, 126}. However, in some studies helminth infection shows no correlation with reduced atopy, and may even demonstrate a positive correlation^{127, 128}. A recent review of helminth-allergy studies performed between 1962 and 2006 (comprising 25,753 individuals) indicates that while hookworm appears to reduce asthma or wheeze, *Ascaris lumbricoides* may exacerbate it¹²⁹. Furthermore, several helminths actually induce allergic reactions themselves, including *Anisakis*, *Echinococcus*¹³⁰ and *Ancylostoma caninum* (dog hookworm)¹³¹. It has been suggested that a prolonged, heavy infection eventually induces T-regulatory cells and IL-10,

providing protection against allergies, whereas a light, transient infection stimulates Th2 responses, exacerbating allergy¹³². This hypothesis may explain why *Anisakis* causes allergies, as this parasite is not adapted to living in humans, and infection is usually transient and involves one to several larvae but no adult worms. In our animal model, *Anisakis* infection was similarly transient and consisted of only two larvae; this protocol resulted in potent Th2 responses and type 2 antibody production. While IL-10 was produced in the early stages of infection, it then subsided and did not show a secondary late increase in response to the release of dead larval proteins like IL-4, IL-5, IL-9 and IL-13. The differential response to live larva and protein products suggest the route of exposure to *Anisakis* allergens may play a role in the different immunological responses elicited. Future studies will aim to isolate *Anisakis pegreffii* allergens, and to determine whether different routes of exposure result in different profiles of allergen recognition, with possible resulting differences in clinical outcome.

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CHAPTER THREE

***ANISAKIS*-INDUCED ANAPHYLAXIS AND THE ROLE OF
IL-4 RECEPTOR ALPHA EXPRESSION ON CD4⁺ T CELLS
AND MACROPHAGES/NEUTROPHILS IN
ANAPHYLACTIC REACTIONS**

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CHAPTER 3: *Anisakis*-induced anaphylaxis and the role of IL-4 receptor alpha expression on CD4⁺ T cells and macrophages/neutrophils in anaphylactic reactions

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3.1. SUMMARY

Background: The fish parasite *Anisakis* is an important cause of anaphylaxis in populations in which large quantities of fish are consumed. Reactions to *Anisakis* are thought to be caused only by live larvae. Therefore, a model was set up to compare *Anisakis*- and ovalbumin (OVA)-induced anaphylaxis in mice. IL-4 and IL-13, which both signal via the IL-4R α , have been shown to exacerbate anaphylaxis, but the cell-specific requirements for IL-4R α expression are not known. This study investigates the relative importance of IL-4R α expression on macrophages and T-cells in systemic anaphylaxis.

Methods: Wildtype mice or those possessing a null mutation (IL-4R α $-/-$), T cell specific (Lck^{cre}IL-4R α ^{-/lox}), or macrophage specific (LysM^{Cre}IL-4R α ^{-/lox}) deletion in IL-4R α were sensitized by intraperitoneal injection at days 0 and 14 with *Anisakis* extract or OVA adsorbed to alum, and challenged intravenously at day 21 with *Anisakis* extract or OVA. Anaphylactic scores, rectal temperatures, gut pathology, mast cell proteases, antibodies and cytokine production were evaluated.

Results: *Anisakis* proteins were able to cause anaphylaxis in mice, comparable to that caused by OVA. OVA-induced anaphylaxis was found to be dependent upon the Fc γ RII/III and associated with mast cell degranulation. Expression of the IL-4R α on T-cells, but not macrophages or neutrophils, was required for systemic reactions. T-cell specific IL-4R α knockout mice demonstrated both Th1 type and Th2 type responses, but were protected against anaphylaxis. IFN- γ was found to be crucial for this protection, as its depletion led to high rates of mortality and exacerbated intestinal pathology.

Conclusions: *Anisakis* proteins may cause anaphylactic reactions in sensitized individuals. IFN- γ serves an important protective role during the effector phase of systemic anaphylaxis, possibly by direct inhibition of mast cell degranulation.

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3.2 INTRODUCTION

Anaphylaxis is a severe, systemic immediate hypersensitivity reaction affecting multiple organ systems, and is potentially fatal. Severe anaphylaxis affects 1-3 in 10 000 people, and the risk of death from anaphylaxis is approximately 1%^{1, 2}. The recent rise in the prevalence of allergy has been accompanied by an increase in the incidence of anaphylaxis³. An Australian study found that 1 in 170 schoolchildren had experienced anaphylaxis⁴. Common causes of anaphylaxis include insect stings, food and drugs^{1, 5}. Peanuts and tree nuts cause the majority of fatalities due to food-induced anaphylaxis⁶⁻⁸. Other foods often implicated in causing anaphylactic reactions include fish, seafood, egg and fruits⁷. However, in certain regions the parasitic nematode *Anisakis* is also an important cause of anaphylaxis^{9, 10}. In an epidemiological study conducted in Spain, *Anisakis* was found to be the third most common cause of anaphylactic reactions, following drugs and Hymenoptera stings and preceding food¹¹.

3.2.1 Symptoms of anaphylaxis

A wide range of anaphylactic reactions are possible, depending to some extent on the route of allergen exposure. Symptoms of anaphylaxis can involve the skin and cardiovascular, respiratory and gastrointestinal systems. They often begin with a metallic taste in the mouth, tingling and warmth, and can proceed to angioedema, urticaria, bronchospasm, diarrhoea, hypotension, shock and/or circulatory failure and cardiac arrest¹². Death may result without rapid injection of epinephrine, and replacement of blood volume with a drip is often required due to the severe extent of vascular leakage. Keeping an anaphylactic patient in the supine position (or left lateral position if vomiting is a symptom) is critical to retain blood flow to the brain, and can prevent death¹³. Corticosteroids and anti-histamines are often co-administered with epinephrine in order to suppress ongoing immune reactions, but there is limited evidence that this is effective. Anaphylaxis accompanied by bronchoconstriction may require inhaled bronchodilators - this is more common in food-induced anaphylaxis. Asthma is associated with an increased risk of death due to anaphylaxis⁷.

3.2.2 Pathology of anaphylaxis

It is accepted that symptoms of anaphylaxis are a result of the release of vasoactive and inflammatory mediators from mast cells and basophils, including histamine, platelet activating factor (PAF), serotonin, leukotrienes and prostaglandins^{1, 5}. Originally only IgE-mediated reactions were termed “anaphylaxis”, while non-IgE mediated reactions were called “anaphylactoid”. However, as the two forms of the disease are clinically the same, the term anaphylaxis is now used to describe both¹⁴. In IgE-mediated anaphylaxis, inflammatory and vasoactive mediators are rapidly released when IgE binds to the FcεRI receptor on the surface of mast cells or basophils, triggering degranulation (Fig.3.2.1). Non-IgE mediated mechanisms of mediator release can involve IgG or complement or even direct stimulation of mast cells or basophils. Sometimes the cause is unknown, in which case it is known as idiopathic anaphylaxis.

The release of anaphylactic mediators causes vasodilation, increased vascular permeability, increased mucosal secretions and smooth muscle contraction.¹³ Dilatation of arteries and arterioles causes increased blood flow to the organs, resulting in reddening. Fluid leaves permeable vessels through openings in intracellular junctions caused by vasoactive mediators and enters surrounding tissue. This vascular leakage causes local oedema, which is especially dangerous when it affects the larynx and lungs as it may cause asphyxiation, as can bronchospasm and mucus plugging of the airways^{8, 13}. Due to fluid extravasion, blood flow through the organs is slowed and congestion and pooling of blood occurs, leading to a build-up of deoxygenated blood and a blueish tinge, known as cyanosis. Venous congestion can enhance the escape of fluid or even red cells to the surrounding tissue due to increased pressure on the vessel walls. In addition, the anoxia resulting from slowed circulation increases vascular permeability. Anaphylaxis is therefore a self-perpetuating and progressive condition. Blood pressure and temperature drop, and ultimately circulatory failure can occur.

3.2.3 *Anisakis*-induced anaphylaxis

Anaphylaxis due to *Anisakis* is currently considered to be primarily caused by an acute infection, with removal of the worm (if visible) forming part of the treatment. The disease is therefore termed “gastroallergic anisakiasis”. Symptoms such as urticaria, angioedema, bronchospasm and/or anaphylactic shock predominate, with or without gastro-intestinal discomfort^{9, 15, 16}. The disease is rarely fatal, but can be severe and extended hospitalisation may be required¹⁵. In oral challenge studies, ingestion of lyophilized larvae or larval antigens did not cause reactions in *Anisakis*-allergic individuals, and many patients who have experienced gastroallergic anisakiasis can continue to eat deep-frozen or well-cooked fish without reactions¹⁷⁻¹⁹. Therefore many physicians consider a live infection necessary to elicit allergic reactions. The allergic symptoms are proposed to be a combination of a memory response to a prior infection (as *Anisakis*-specific IgE is typically present) and an acute inflammatory response to a current infection¹⁵. However, as live larvae are not always detected, it cannot be ruled out that *Anisakis* proteins alone can cause anaphylaxis in the manner of a conventional food allergen such as egg or peanut. The fact that anaphylaxis can occur after skin-prick tests for *Anisakis* illustrates this possibility²⁰, and angioedema/anaphylaxis has occurred after the ingestion of cooked fish and pizza made with canned tuna^{21, 22}. In the previous chapter it was shown that infection with live larvae strongly predisposed mice to subsequent allergic reactions upon ingestion of *Anisakis* proteins. This chapter investigates whether *Anisakis* proteins alone are able to cause anaphylaxis in a traditional murine model of anaphylaxis, using the model allergen chicken egg ovalbumin (OVA) as a positive control.

3.2.4 Mouse models of anaphylaxis

Experimental models of anaphylaxis have illustrated that two pathways of anaphylaxis exist in mice: one mediated by IgE and the other by IgG²³⁻²⁸ (Fig. 3.2.1). IgE-dependent anaphylaxis is dependent on antigen-crosslinking of IgE bound to the FcεRI receptor on mast cells, and subsequent release of mediators such as histamine, serotonin and PAF^{24, 25, 29}. Systemic shock in this system is believed to be mediated predominantly by histamine, with PAF playing an exacerbating role^{24, 25}, although in penicillin V induced anaphylaxis, PAF-inhibitor was able to completely block reactions²⁷. Intestinal reactions

and diarrhoea are histamine-independent but dependent upon serotonin and PAF released by mast cells^{25,30,31}. The release of mouse mast cell protease-1 (MMCP-1) also increases intestinal permeability³².

IgG-dependent anaphylaxis can occur independently of mast cells in a mechanism dependent on macrophages, FcγRIII and PAF²³⁻²⁵. Whether IgE- or IgG-dependent anaphylaxis occurs appears to be determined by the quantity of antigen-specific IgG present and the challenge dose used²⁵. The fact that human anaphylaxis is usually IgE-dependent may therefore be due to the low concentrations of antibody and antigen that are usually involved. In mice, antigen-specific IgG can inhibit IgE-mediated anaphylaxis when both IgG and IgE are present³³. This blocking effect is only determined by measuring mast cell degranulation, as physical effects are masked by the fact that IgG can induce anaphylaxis itself. In humans, the ratio between IgG4 and IgE is thought to determine whether allergic reactions occur, as these isotypes may compete for the same antigenic targets³⁴. As cross-linking of bound IgE molecules is responsible for degranulation of mast cells and basophils, the presence of IgG4 may cause a physical interference or reduce the amount of free antigen.

It is not known whether the FcγRIII/macrophage pathway exists in humans. *In vitro*, antigen-specific IgG is able to cause human basophil/mast degranulation and release of mediators^{35,36}. The same mediators are released by both IgG- and IgE-mediated degranulation³⁶. Furthermore, IgG-mediated anaphylactic reactions have been reported in humans³⁷⁻⁴⁰. Clinically, IgE-mediated and IgG-mediated reactions are virtually identical¹⁴. Similarly, in mice the outcome of IgE-mediated and IgG-mediated pathways is much the same, with involvement of the same target organs²⁴. Therapies relevant to mice may therefore have possible application in humans.

The Th2 cytokines IL-4 and IL-13 play an important role in both IgE- and IgG-mediated anaphylaxis in a STAT6 and IL-4Rα dependent manner⁴¹. These cytokines both influence the initial accumulation of antibody and effector cells, and enhance the biological effects of vasoactive mediators. Accordingly, mice deficient in the IL-4Rα,

through which both cytokines signal, have less severe anaphylaxis. However, the role of IL-4R α expression on specific cell-types is unknown. For this reason the present study evaluates antigen-induced anaphylaxis in T-cell specific and macrophage/neutrophil specific IL-4R α deficient mice that have been previously characterised by our research group^{42, 43} (see Chapter One). IL-4 signalling via the IL-4R α in CD4⁺ T cells is thought to be critical for the development of Th2 effector cells that promote a Th2 type cytokine environment^{44, 45}. Alternative macrophage activation is known to be induced by IL-4/IL-13⁴⁶, and the role of alternative macrophages in acute allergic diseases is unknown.

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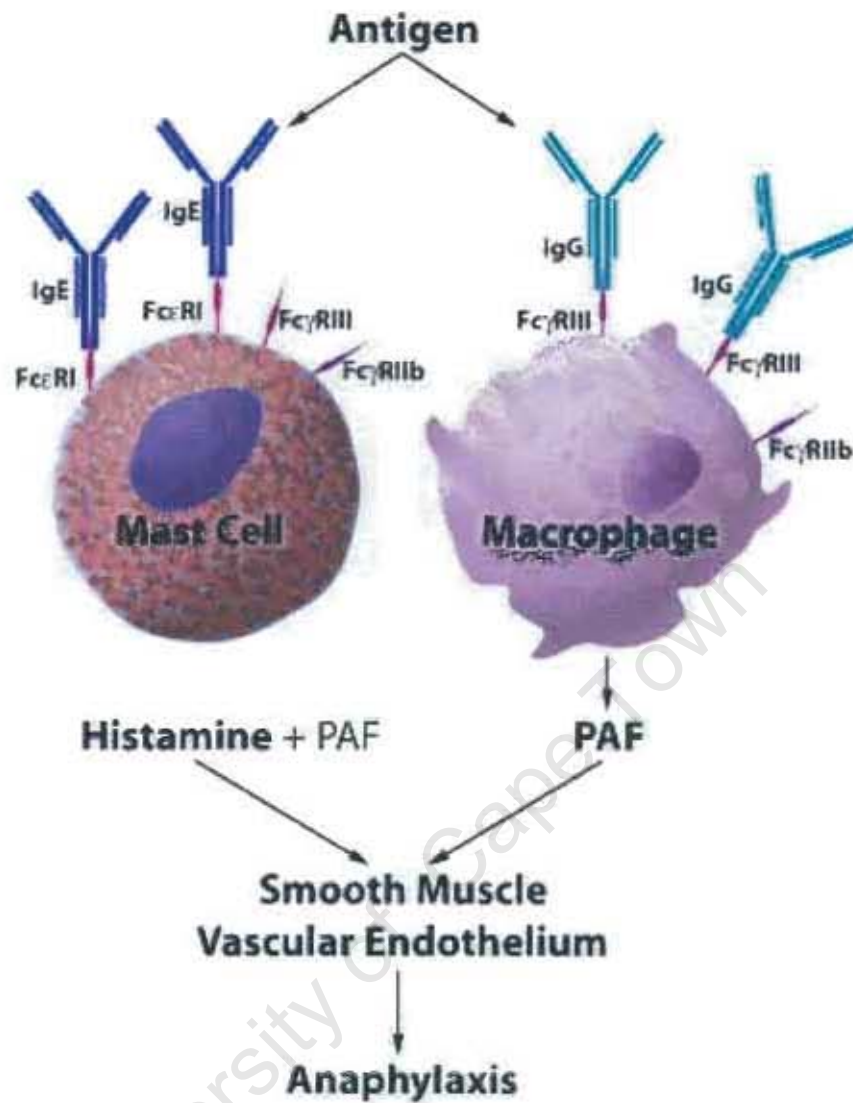


Figure 3.2.1. Current model of anaphylactic pathways in mice. This figure from Finkelman et al.²⁵ outlines pathways of anaphylaxis that have been experimentally demonstrated in mice. In the first pathway (left), allergen binding cross-links IgE bound to the FcεRI receptor on mast cells, causing mast cell degranulation and the release of the vasoactive mediators histamine and platelet-activating factor (PAF). Histamine is the central mediator of anaphylaxis in this pathway. In the second pathway (right), allergen-IgG complexes binding to FcγRIII on the surface of macrophages cause the release of PAF.

3.3 METHODS

Mice

BALB/c wildtype mice and IL-4R α $-/-$ mice⁴⁷, macrophage/neutrophil specific IL-4R α $-/-$ (LysM^{Cre}IL-4R α ^{-/lox})⁴² and CD4⁺ T-cell specific IL-4R α $-/-$ (Lck^{Cre}IL-4R α ^{-/lox}) mice⁴³ on a BALB/c background were housed under SPF barrier conditions using individual ventilated cages (IVC). All experiments complied with the South African Code of Practice, and were approved by the University of Cape Town's Animal Ethics Committee.

Anisakis extract

Anisakis pegreffii larvae (L3) were collected from the intestines of parasitized fish (*Thyrsites atun*) with forceps, and extensively washed in 4% acetic acid and phosphate-buffered saline (PBS) (see Appendix A). The larvae were frozen, homogenized in PBS and sonicated with a MicrosonTM ultrasonic cell disrupter (Misonix)(Long Island, U.S.A.) to lyse cells. The extract was pipetted into 2ml eppendorf tubes and centrifuged at 14000rpm for 20-30 minutes to remove particulate matter, then filter-sterilized through a 0.20 μ m filter (Sartorius, Goettingen, Germany). Protein concentration was measured by BCA test (Pierce, Rockford, Illinois, U.S.A.) according to the manufacturer's protocol.

Sensitization and challenge protocol

Mice were injected intraperitoneally with 50 μ g ovalbumin grade V (Sigma, Steinheim, Germany) or *Anisakis* extract adsorbed to 0.6mg/ml aluminum hydroxide gel (Sigma) in 200 μ l of PBS at day 0. The same preparation was used for the boost at day 14. At day 21, mice were challenged intravenously with 200 μ l ovalbumin or *Anisakis* extract. Doses of 500 μ g, 750 μ g or 1mg were initially tested before a dose of 750 μ g was settled upon for use in all experiments. Mice that survived challenge were anaesthetised with 200 μ l of ketamine (Anaket-V; Centaur Labs, Johannesburg, South Africa)/xylazine (Rompun; Bayer, Isando, South Africa) anaesthetic (see Appendix A) in order to take blood and were killed by cervical dislocation.

Anaphylaxis score

Mice were observed up till 70 minutes post challenge, and signs of anaphylaxis were scored using a modification of a previously described scoring system⁴⁸. 0, no symptoms; 1, hypersensitivity to touch, irritability/aggression; 2, diarrhoea, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate; 3, weakness, cyanosis around mouth and tail, lying flat but upright; 4, lying sideways, no activity upon prodding, loss of grip, loss of consciousness, tremor or convulsions; 5, death. Mice experiencing convulsions invariably died soon after, and were therefore euthanased for humane reasons and scored as 5.

Temperature measurement

Rectal temperature readings were taken every 10 minutes using a mouse rectal thermometer (VetTech, Cheshire, U.K.) lubricated with VaselineTM petroleum jelly (Unilever, La Lucia, South Africa).

Antibody depletion

The following rat anti-mouse antibodies were purified by affinity chromatography using Protein G Sepharose (Sigma, Steinheim, Germany) and used in antibody depletion experiments: anti-CD4 (GK1.5), anti-IL-4 (11B11), anti-IgE (EM95.3 or the other), anti-Fc γ RII/III (2.4G2), anti-IFN- γ (ANI18KL6), anti-ckit (ACK-2) provided by Dr. Richard Grencis (University of Manchester) and control rat IgG (GL113). For IgE, Fc γ RII/III and IFN- γ depletion, mice were injected intraperitoneally with 1mg of antibody at days 18 and 20. For anti-IL-4 and anti-CD4 depletion mice were injected intraperitoneally with 1mg of antibody at days 0 and 14. For anti-ckit depletion mice were injected intraperitoneally with 1mg of antibody at day -1, day -1 and 3, or day -1, 3 and 14.

Collection of serum

Blood samples taken before challenge were collected in serum separator tubes (BD MicrotainerTM SST, BD, Franklin Lakes, U.S.A.) by tail vein bleeding under an infra-red heating lamp. Alternatively, blood was taken from killed mice after cervical dislocation.

Blood samples were centrifuged for 15 minutes at 3000 rpm before being placed at -80°C for storage.

Antigen-specific antibody ELISAs

Nunc Maxisorp ELISA plates (Nunc, Reskilde, Denmark) were coated overnight at 4°C with *Anisakis* extract or ovalbumin grade V (Sigma, Steinheim, Germany) at 5µg/ml (for IgG1, IgG2a, IgG2b) or 1mg/ml (for IgE) in PBS, then blocked in ELISA blocking buffer (Appendix A) for 1 hour at 37°C. Plates were washed 3x with ELISA wash buffer (Appendix A), and serum samples serially diluted in ELISA Dilution Buffer (Appendix A) were added. Plates were incubated overnight at 4°C, then washed 4x. Alkaline-phosphatase (AP)-labelled goat-anti-mouse IgG1, IgG2a, IgG2b or IgE, 0.5µg/ml, (Southern Biotechnology, Birmingham, Alabama, U.S.A.) was added for 2 hours at 37°C. Plates were washed 4x and 4-nitrophenyl phosphate disodium salt hexahydrate (PNP) (Sigma, Steinheim, Germany) substrate was added (Appendix A). Absorbance was measured at 405nm with 492nm as a reference wavelength using an ELISA reader (VERSAmax tuneable microtitre plate reader, Molecular Devices, Sunnyvale, California, U.S.A.).

Total IgE ELISAs

Sandwich ELISA was performed using purified anti-IgE from clone 84.1C as a coat (1µg/ml) and AP-labelled goat anti-mouse IgE (Southern Biotechnology, Birmingham, Alabama, U.S.A.) as a secondary antibody, according to the protocol outlined above ("Antigen-specific antibody ELISAs"). Purified recombinant mouse IgE (BD Pharmingen, San Diego, U.S.A) was used as a standard, starting at a concentration of 10µg/ml.

Mouse mast cell protease (MMCP)-1 ELISAs

MMCP-1 levels were measured in serum and tissue homogenates by ELISA according to the manufacturer's protocol (Moredun Scientific Ltd, UK). For serum MMCP-1, blood was centrifuged in separator tubes (manufacturer) for 15 minutes at 4000rpm to isolate serum. For gut MMCP-1, pieces of gut were removed, weighed and homogenized in

PBS, then centrifuged at 12000rpm for 20 minutes to remove debris. Supernatants were removed for analysis and the results were adjusted for organ weight.

Isolation of splenocytes

Spleens were aseptically removed from mice with forceps, and splenocytes were isolated by pressing through a metal sieve in 10ml Iscove's Modified Dulbecco's Medium (IMDM)(Gibco, Paisley, Scotland). The cells were centrifuged at 1200rpm for 5 minutes, and the pellet was resuspended in 3-5ml of ice-cold red cell lysis buffer (Appendix A). After 2-5 minutes on ice, the cells were centrifuged at 1200rpm for 5 minutes. Pelleted cells were resuspended in 10ml of IMDM supplemented with 10% FCS (Delta, Kempton Park, South Africa), 2mM L-glutamine (Gibco, Paisley, Scotland), 100µg/ml penicillin (Gibco, Paisley, Scotland) and 100µg/ml streptomycin(Gibco, Paisley, Scotland). The splenocytes were then filtered through a 70µm cell strainer (BD Falcon, Bedford, Massachusetts, U.S.A.) to remove debris, and diluted 1 in 10 to 1 in 30 with Trypan Blue (10µl)(Sigma, Steinheim, Germany) and PBS for counting.

Splenocyte restimulation

Splenocytes were diluted to 4×10^6 cells/ml with IMDM (Appendix A), and cultured in 48-well plates (Costar, Corning, New York, U.S.A.), with 250µl of cells added to one of the following: 250µl medium, wells pre-coated with anti-CD3 (clone 145-2C11; 10µg/ml) overnight at 4°C to which 250µl of medium had been added, 25µg of conA in 250µl medium, 250µl of 200µg/ml *Anisakis* extract in medium, 250µl of 200µg/ml Grade V ovalbumin (Sigma, Steinheim, Germany) in medium. The cells were incubated at 37°C with 5% CO₂, and supernatants were collected after 48 hours and stored at -80°C.

ELISPOTS

Multiscreen plates (Millipore, Cork, Ireland) were coated overnight at 4°C with 5µg/ml rat-anti mouse IL-4 (clone 11B11, 25µg/ml) or rat anti-mouse IFN-γ (R46A2), in PBS (see Appendix A), then washed 3x with sterile ELISA wash buffer (Appendix A), patted dry, and blocked for 1 hour at 37°C with 5% BSA/PBS (filter-sterilized). Plates were

washed 3x, patted dry, then incubated for 10 minutes in IMDM at room temperature. The medium was removed, and splenocytes in IMDM were added at a concentration of 5×10^5 cells per well, then incubated at 37°C for 40-48 hours. Cells were then removed by washing 9x in ELISA wash buffer (Appendix A) and 1x in distilled water. The plates were incubated for 3 hours at room temperature with 0.5 µg/ml biotinylated anti-IL-4 or anti-IFN-γ (BD Pharmingen, San Diego, U.S.A.) diluted in 1% BSA/PBS). Then the plates were washed 6x, patted dry and incubated for 2 hours at room temperature with 0.5 µg/ml AP-labelled streptavidin (BD Pharmingen, San Diego, U.S.A.), following which plates were washed 5x with ELISA wash buffer and 1x with PBS. Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) (Sigma, Steinheim, Germany), made up according to the manufacturer's protocol, was added. The plates were incubated 5-30 minutes until the desired colour change was achieved. The reaction was stopped by washing 3x with distilled water, plates were allowed to air-dry, and the spots were counted using a dissecting microscope.

Cytokine ELISAs

Sandwich ELISAs were performed to determine cytokine levels in cell supernatants. Nunc Maxisorp microtitre plates (Nunc, Reskilde, Denmark) were coated with purified anti-IL-4 (clone 11B11, 2 µg/ml), anti-IFN-γ (clone An18KL6, 1 µg/ml) or anti-IL-5 (1 µg/ml), anti-IL-9 (2 µg/ml), anti-IL-13 (1 µg/ml), anti-IL-10 (1 µg/ml) or anti-TGF-β (0.5 µg/ml) (all BD Pharmingen) diluted in PBS, and incubated overnight at 4°C. Plates were blocked with ELISA block buffer (Appendix A) for 1-2 hours at 37°C, then washed 3x with ELISA wash buffer (Appendix A). Serially diluted standards (purified recombinant IL-4, IL-5, IL-9, IL-13, IFN-γ, IL-10 or TGF-β (all BD Pharmingen, San Diego, U.S.A.) or cell supernatant, all diluted in ELISA dilution buffer (Appendix A) were added to the plates, and incubated overnight at 4°C. Samples for the detection of TGF-β were pre-treated with acid to unfold the protein for antibody binding to unfold the protein for antibody binding (Appendix B). The plates were washed 4x, and biotinylated goat-anti-mouse IL-4 (0.5 µg/ml), IL-5 (0.5 µg/ml), IL-9 (1 µg/ml), IL-13 (0.5 µg/ml), IFN-γ (0.5 µg/ml), IL-10 (0.5 µg/ml) or TGF-β (0.5 µg/ml) antibodies (all BD Pharmingen, San

Diego, U.S.A.) diluted in ELISA dilution buffer were added for 3 hours at 37°C. Plates were washed 4x and 1 µg/ml AP-labelled streptavidin (Pharmingen) was added for 1 hour at 37°C. Finally, plates were washed 4x and (PNP) (Sigma, Steinheim, Germany) substrate (see Appendix A) was added. Absorbance was measured at 405nm with 492nm as a reference wavelength using a VERSAmax tuneable microplate reader (Molecular Devices, Sunnyvale, California, U.S.A.). Cytokine levels in supernatants were determined by reading from the standard curve.

Statistical analysis

Values are given as mean ± SEM and significant differences were determined using the unpaired two-tailed Student's t-test using computer software (GraphpadPrism™). For allergic score graphs, the Mann-Whitney test was used for two experimental groups, and the Kruskal-Wallis test with Dunn's post test for three or more groups. Values P < 0.05 were considered significant.

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3.4 RESULTS

3.4.1 *Anisakis* proteins can cause anaphylaxis in a murine model

Using previous publications as a guideline^{49, 50} a model of antigen-driven anaphylaxis was set up using *Anisakis* crude extract, OVA as a positive control or PBS as a negative control. Wild-type and IL-4/IL-13 deficient mice were sensitized with two intraperitoneal, PBS/alum, *Anisakis*/alum or OVA/alum injections, and then challenged intravenously with *Anisakis* or OVA in PBS (Fig. 3.4.1).

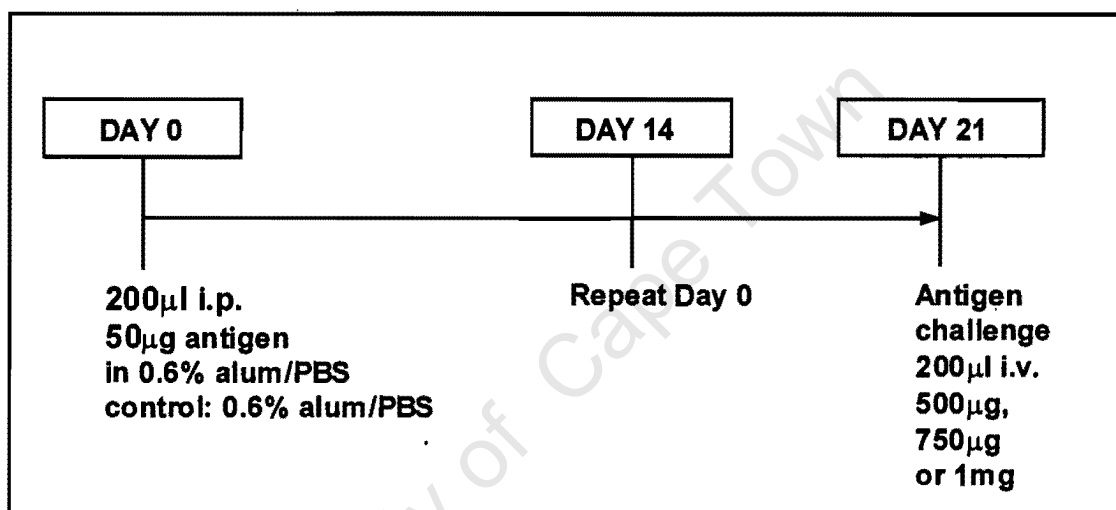


Figure 3.4.1. Sensitization and challenge protocol.

The rectal temperatures of both *Anisakis* and OVA-sensitized wild-type mice began to drop within ten minutes of intravenous injection, while PBS sham-sensitized mice maintained relatively constant temperatures. (Fig. 3.4.2). Hypothermia was accompanied by a loss of activity, but did not always correlate with the severity of anaphylaxis, which was better evaluated using a scoring system (see Methods). Mice experienced systemic symptoms ranging from diarrhoea and decreased activity to cyanosis, loss of consciousness and death. The presence of intestinal oedema and hyperaemia, likely driven by mediators of vascular permeability and vasodilation, was associated with certain fatality (Fig. 3.4.3a). Circulatory failure due to hypovolaemic shock was the likely cause of death in these mice. Figure 3.4.3b shows the intestines of a control-sensitized and an OVA-sensitized mouse after intravenous injection of OVA combined with Evan's

Blue. The dark blue colour in the antigen-sensitized mouse indicates the vascular leakage caused by the challenge.

Anisakis sensitized and challenged IL-4/IL-13 $-/-$ mice experienced a smaller decrease in temperature than wild-type mice, accompanied by a substantial decrease in symptom severity (Fig. 3.4.2). However, OVA-sensitized and challenged IL-4/IL-13 $-/-$ mice showed hypothermia and reactions of approximately the same degree as wild-type mice. Anaphylaxis on the whole was more severe in OVA- challenged mice than in *Anisakis*-challenged mice, with mice succumbing more rapidly to shock. Based on the severity of the reactions, even in the IL-4/IL-13 $-/-$ mice which are typically used for a negative phenotype in allergy experiments, it was decided that a lower challenge dose would be preferable.

3.4.2 Dose-dependent responses in wild-type and IL-4R α $-/-$ mice

Challenge doses of 500 μ g and 750 μ g of *Anisakis* extract were compared in wild-type and IL-4R α $-/-$ mice (Fig. 3.4.4). The IL-4R α has been shown to serve an essential role in various models of allergy, and IL-4 and IL-13, which both signal through this receptor, have been shown to exacerbate anaphylaxis^{25, 41}.

When challenged with 500 μ g of *Anisakis* extract, IL-4R α mice did not develop hypothermia or show signs of anaphylaxis. In contrast, when dosed with 750 μ g of *Anisakis* extract, IL-4R α $-/-$ mice showed a temporary decrease in rectal temperature and activity, but did not demonstrate the severe shock phenotype of the wild-types. During the post-mortem, the intestines of IL-4R α mice appeared normal and there were

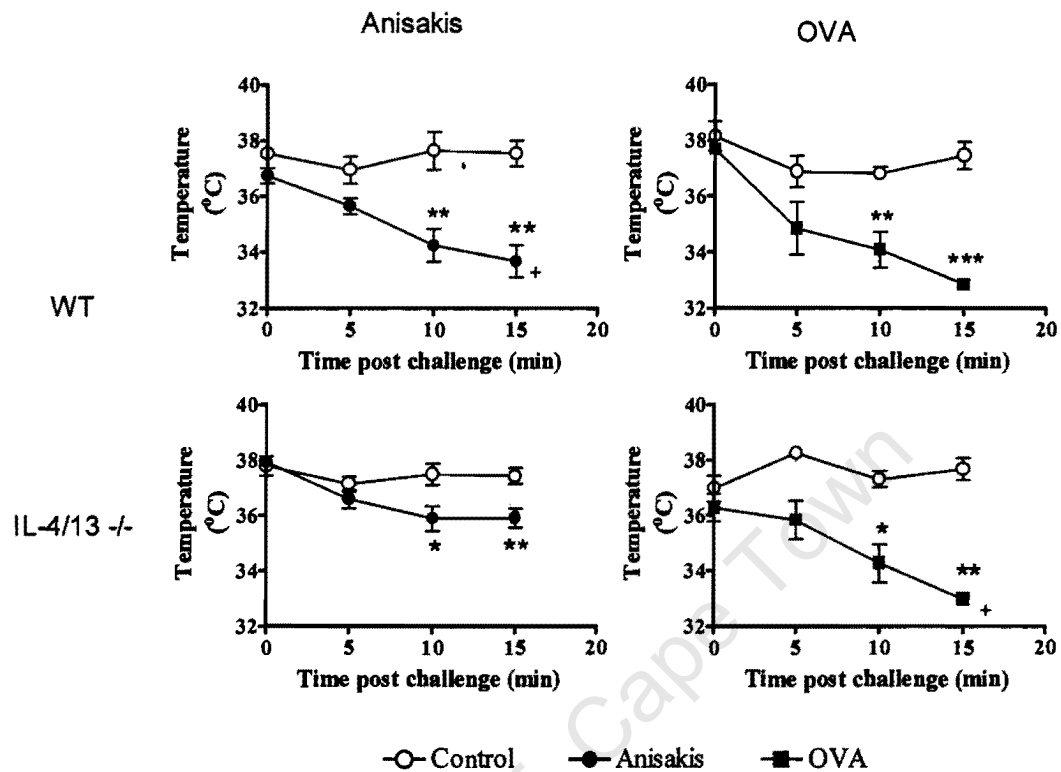


Figure 3.4.2. Antigen-induced anaphylaxis in wildtype and IL-4/IL-13 $-/-$ mice. Mice were sensitized intraperitoneally at day 0 and 14 with 50 μ g of *Anisakis* extract or OVA adsorbed to alum, and challenged intravenously at day 21 with 1mg of *Anisakis* extract or OVA. Rectal temperatures were measured over 15 minutes. Data presented as mean \pm SEM. Statistical significance between control and antigen-challenged mice is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, + death of a mouse.

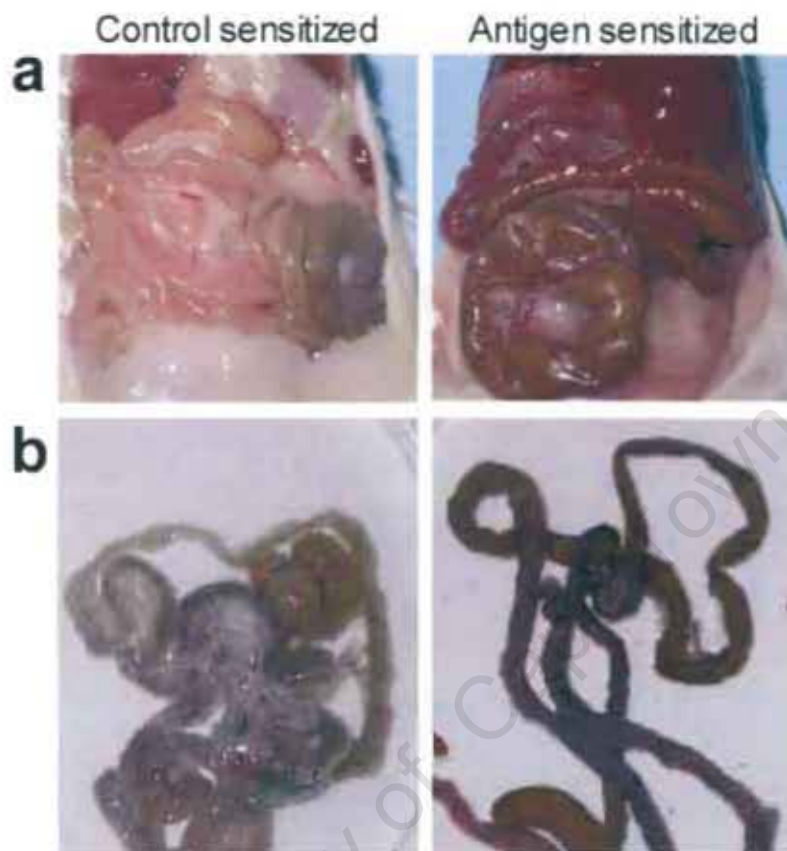


Figure 3.4.3. Vascular leakage after antigen challenge in wildtype mice.

a) Intestinal odema and hyperaemia occurs in antigen-sensitized mice (right) but not PBS-sensitized mice (left) challenged intravenously with antigen. **b)** Intestines from Evan's Blue injected mice. Antigen for challenge was administered in a 1% Evan's Blue dye solution in order to monitor vascular permeability. In antigen-sensitized mice (right) the intestines are coloured dark blue, illustrating vascular leakage and exudation of Evan's Blue dye.

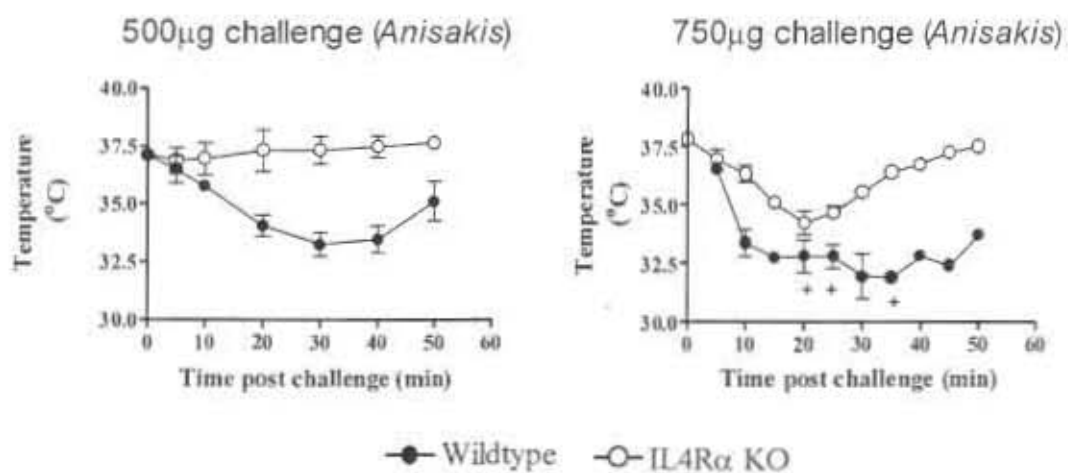


Figure 3.4.4. Challenge dose comparison in wildtype and IL-4R α $-/-$ mice. Rectal temperatures were measured post intravenous challenge with 200µl of *Anisakis* extract. +, death of a mouse. Data presented as mean \pm SEM.

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no signs of the oedema or vasodilation that was found in wildtype mice. This intermediate phenotype was clearly distinguishable from the more severe phenotype of the wildtype mice, but left a margin for studying ways to completely abrogate anaphylaxis in future experiments. The 750 μ g dose was therefore used in subsequent experiments.

3.4.3 IL-4R α expression on CD4⁺ T-cells plays a crucial role in anaphylaxis

While it has been previously shown that the IL-4R α plays an important role in anaphylaxis²⁵, as it does in our model (Fig. 3.4.4), the cell-specific requirements for IL-4R α expression are not known. The availability of CD4⁺ T-cell specific IL-4R α $-/-$ mice in our laboratory enabled us to dissect the role of IL-4R α on CD4⁺ T-cells in antigen-induced anaphylaxis.

After intravenous challenge, mice which completely lacked IL-4R α once again demonstrated markedly less severe reactions than wildtype mice, with a rectal temperature drop that was both less extreme and shorter-lived (Fig. 3.4.5). Intestinal vasodilation, hyperaemia and oedema was not found in these mice. Interestingly, deletion of the IL-4R α on CD4⁺ T-cells alone also resulted in striking protection against anaphylaxis in both the *Anisakis* and OVA models (Fig. 3.4.5). Often this protection was better than that afforded by complete IL-4R α knockouts. Once again, reactions in all strains were dose dependent. At low challenge doses (500 μ g *Anisakis*), neither IL-4R α $-/-$ nor CD4⁺ T-cell specific IL-4R α $-/-$ mice demonstrated any signs of anaphylaxis (data not shown). At moderate doses (750 μ g *Anisakis*), IL-4R α mice showed a transient temperature drop with recovery, while CD4⁺ T-cell specific IL-4R α $-/-$ mice did not react (Fig. 3.4.5). At high doses (750 μ g OVA), both IL-4R α $-/-$ and CD4⁺ T-cell specific IL-4R α $-/-$ mice showed temperature decrease and signs of anaphylaxis (generally milder in CD4⁺ T-cell IL-4R α $-/-$ mice)(Fig.3.4.5). Over several experiments, CD4⁺ T-cell specific IL-4R α deficient mice consistently demonstrated either complete abrogation of anaphylaxis or transient temperature loss and reduced activity without intestinal pathology as seen in complete IL-4R α knockouts. These data indicate that IL-4R α on

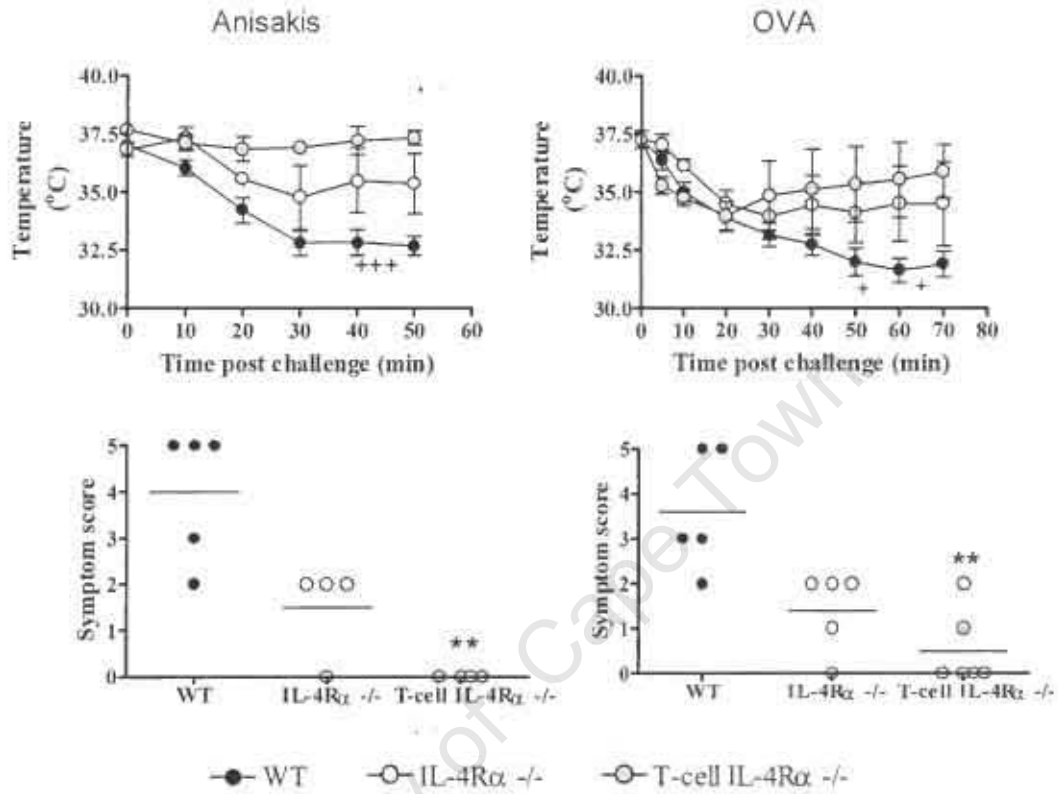


Figure 3.4.5. Anaphylaxis is strongly reduced in CD4⁺ T-cell specific IL-4R α -/- mice. Rectal temperatures were measured after intravenous challenge with 750 μ g of antigen (*Anisakis* extract or OVA). Reactions were scored according to a modification of a previously published symptom scale (see Methods). +, death of a mouse. Data presented as mean \pm SEM.

CD4⁺ T-cells plays a crucial role in mediating anaphylaxis.

3.4.4 CD4⁺ T-cell specific IL-4R α -/- mice exhibit mixed type1/type2 responses

Analysis of antibody isotype production after intraperitoneal sensitization with alum-adsorbed *Anisakis* showed that CD4⁺ T-cell specific IL-4R α -/- mice had raised levels of both the type 2 antibody IgG1 and the type 1 antibodies IgG2a and IgG2b (Fig. 3.4.6), as well as raised total IgE (Fig. 3.4.7). In contrast, wildtype mice produced predominantly type 2 antibodies (IgG1 and IgE) while complete IL-4R α -/- mice produced mainly type 1 antibodies (IgG2a and IgG2b). Total IgE levels followed a similar trend to levels of MMCP-1 in the serum (Fig 3.4.7, 3.4.8), with CD4⁺ T-cell specific IL-4R α -/- mice having lower levels of both than wild-types, and complete IL-4R α -/- mice showing only very low MMCP-1 levels and undetectable total IgE.

ELISPOT analysis (Fig. 3.4.9) demonstrated that the CD4⁺ T-cell specific IL-4R α -/- mice had significant populations of both IFN- γ and IL-4 producing cells, in contrast to wildtypes, in which IFN- γ producing cells were absent. Complete knockouts also possessed both IFN- γ and IL-4 producing cells, though without the IL-4R α would not be able to respond to the IL-4, resulting in the observed type 1 antibody phenotype. The increase in IFN- γ producing cells in *Anisakis*-sensitized CD4⁺ T-cell specific IL-4R α -/- mice as compared to PBS-sensitized controls correlated with a trend towards increased IFN- γ production by spleen cells restimulated with anti-CD3 (Fig 3.4.10). In addition to IFN- γ , anti-CD3 restimulated cells from antigen-sensitized mice produced the Th2 effector cytokines IL-13 and IL-5 in similar amounts to wildtype mice; however, production of IL-4 and IL-9 appeared to be decreased. In OVA-restimulated cells, IL-5 and IL-13 production was also reduced compared to that of splenocytes from wildtype mice.

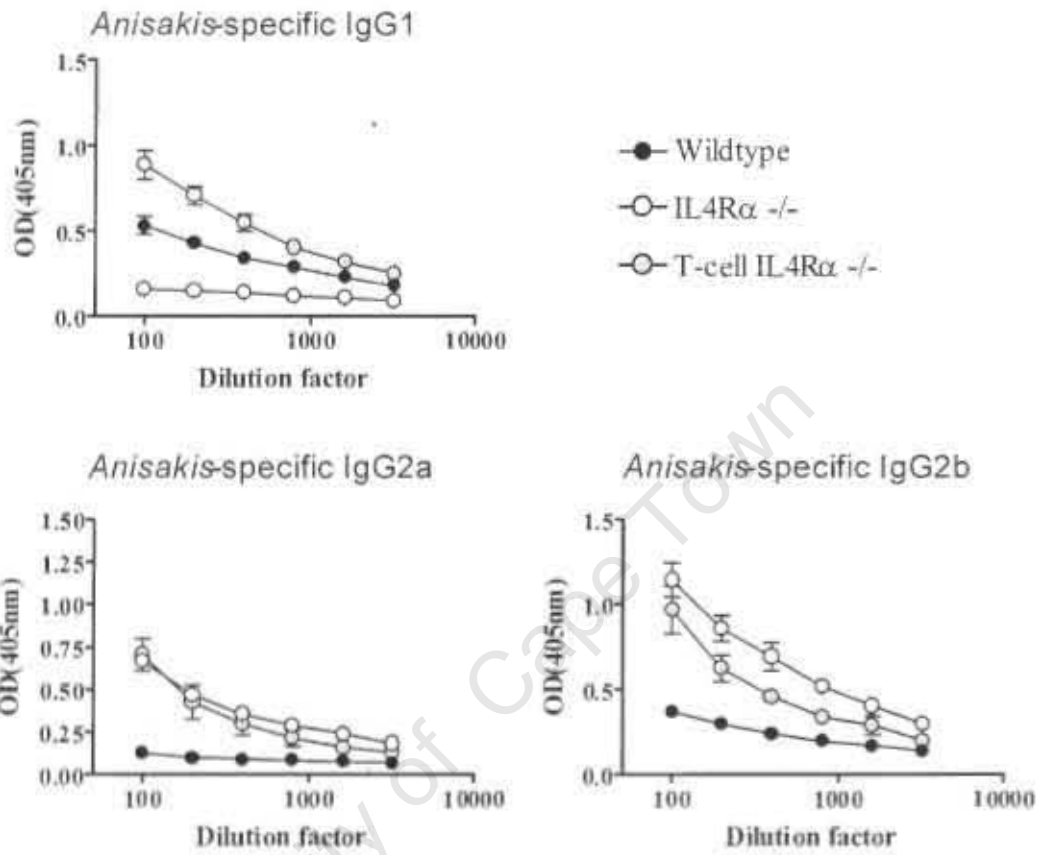


Figure 3.4.6. CD4⁺ T-cell specific IL-4R α -/- mice exhibit mixed type1/type 2 antibody responses. *Anisakis*-specific antibodies were measured at day 21 in *Anisakis*/alum sensitized mice by ELISA. Data presented as mean +/- SEM.

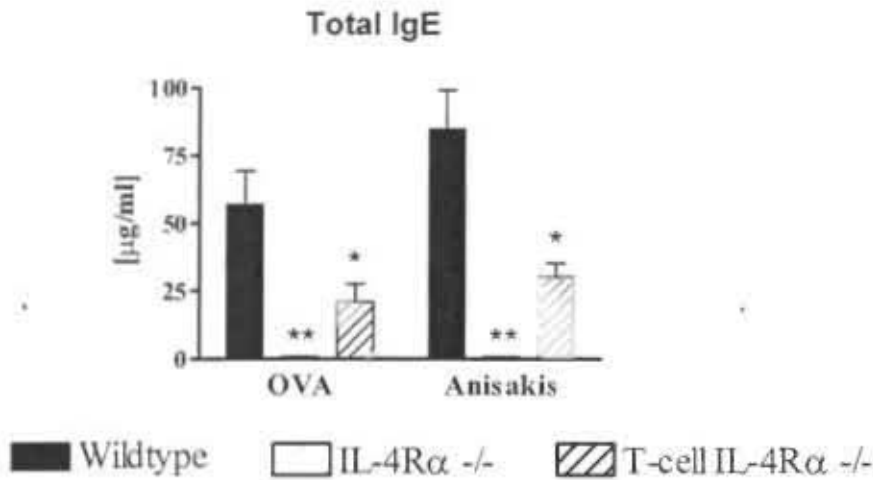


Figure 3.4.7. Total IgE in OVA- and *Anisakis*-sensitized mice. Antibodies were measured in serum taken after the challenge, using ELISA. IgE levels of gene deficient mice were compared to those of wildtype mice. Data presented as mean \pm SEM. Statistical significance between wildtype and knockout mouse strains is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

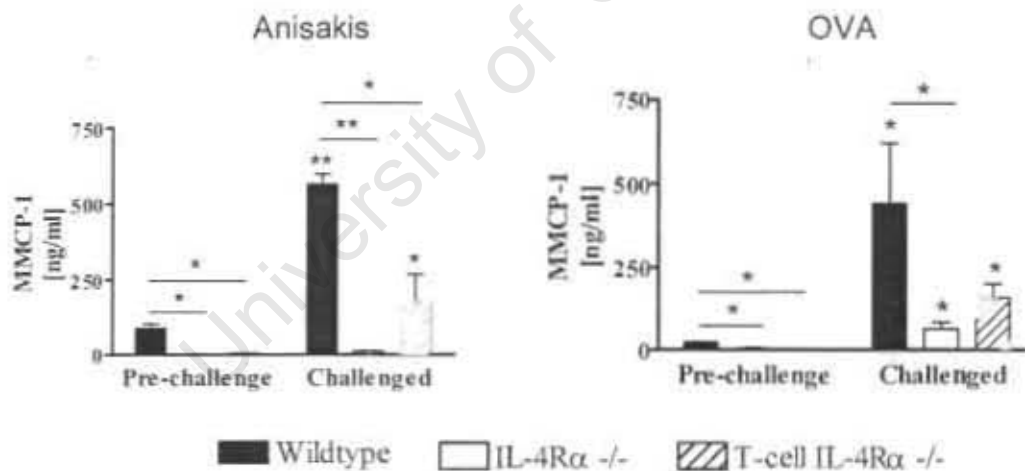


Figure 3.4.8. MMCP-1 levels in serum of *Anisakis* and OVA sensitized mice. Serum MMCP-1 was measured before and after intravenous challenge. Pre-challenge values were compared to post-challenge values, and wildtype mice were compared to gene deficient mice. Data presented as mean \pm SEM. Statistical significance between pre- and post-challenge values is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

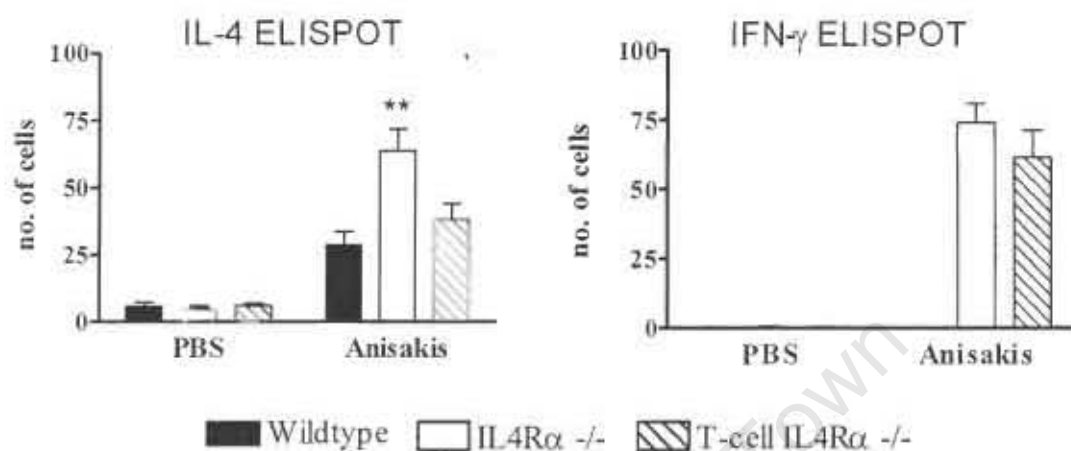


Figure 3.4.9. CD4⁺ T-cell specific IL-4Rα^{-/-} mice have both IL-4 and IFN-γ producing cells. ELISPOTS were performed to detect IL-4 and IFN-γ producing cells amongst plated splenocytes from PBS and *Anisakis*-sensitized mice. Data presented as mean +/- SEM. Statistical significance between PBS and *Anisakis*-challenged mice is shown. **, P<0.01.

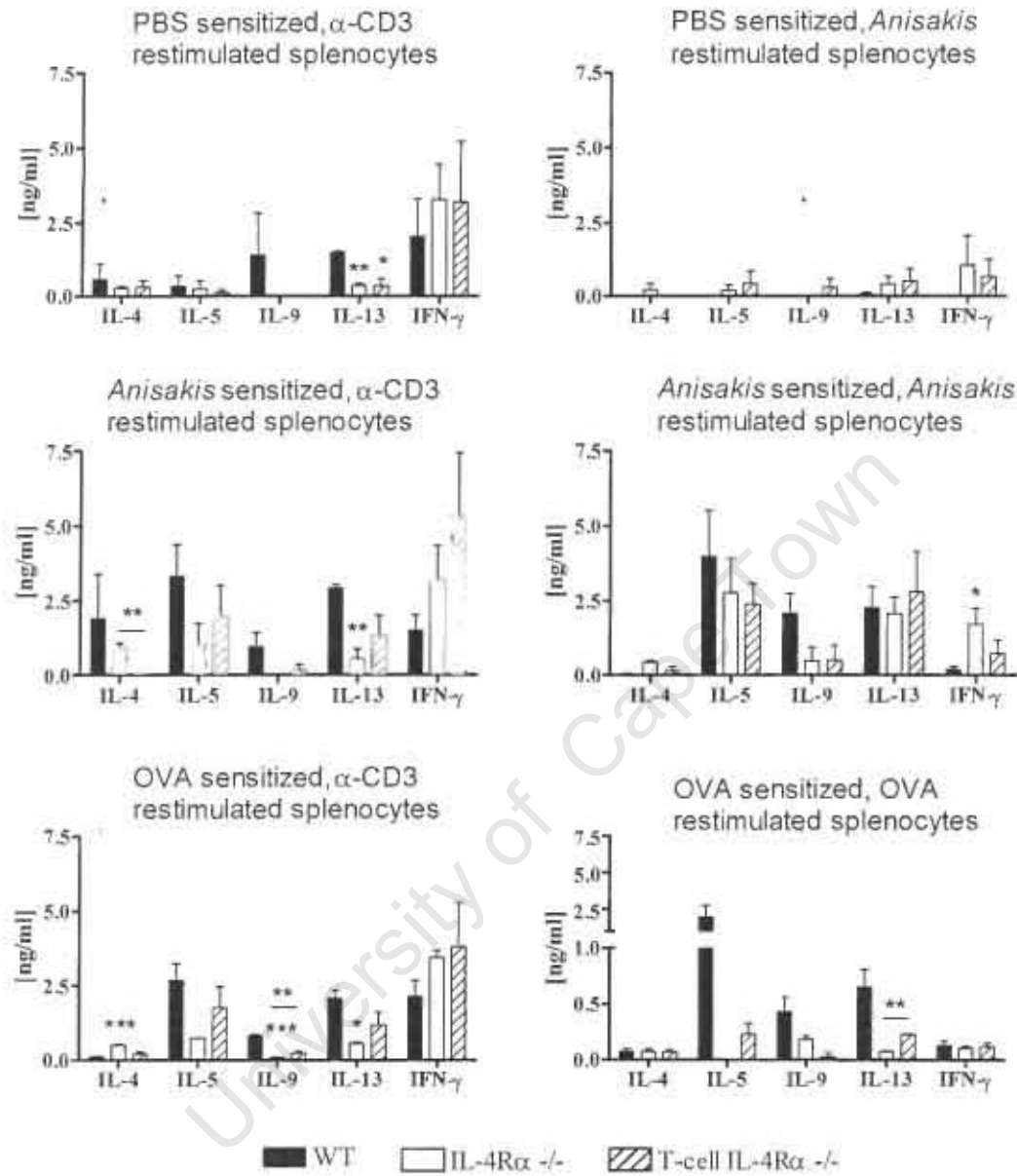


Figure 3.4.10. Cytokine production by restimulated splenocytes in wild-type, IL-4R α -/- and CD4⁺ T-cell specific IL-4R α -/- mice. Splenocytes were isolated on the day of challenge and restimulated with anti-CD3 or antigen (100 μ g/ml *Anisakis* or OVA). Data presented as mean \pm SEM. Statistical significance between wildtype and knockout mouse strains is shown. *, P<0.05; **, P<0.01; ***, P<0.001.

3.4.5 Depletion of CD4⁺ cells reduces the severity of anaphylaxis

Neutralizing antibody was used to deplete CD4⁺ cells at days 0 and 14 using the model of *Anisakis*-induced anaphylaxis. Fig. 3.4.11 shows that anti-CD4 depletion decreased levels of IgG antibodies to barely detectable, although total IgE remained present. In anti-CD4 treated CD4⁺ T-cell specific IL-4R α ^{-/-} mice, total IgE was reduced compared to that of control antibody treated mice.

ELISPOTS demonstrated the presence of many IL-4 producing cells in both treated and untreated mice (too numerous to count, data not shown) while IFN- γ producing cells were almost entirely absent from anti-CD4 treated mice, apart from a small population remaining in the complete IL-4R α ^{-/-} strain (Fig. 3.4.12a). Restimulated splenocytes from all anti-CD4 treated strains did not produce detectable levels of cytokines (data not shown), while cells from control antibody treated strains showed the same pattern of cytokine production as in previous experiments (Fig.3.4.12b). Together these data indicate that CD4⁺ cells are primarily responsible for producing the high IFN- γ levels in CD4⁺ T-cell specific IL-4R α ^{-/-} mice.

Anti-CD4 treated wildtype mice still displayed anaphylaxis after intravenous challenge, though with a decreased degree of severity (Fig.3.4.13). This might be due to maintenance of the IgE-mediated response, as demonstrated in Fig. 3.4.11, since in IL-4R α ^{-/-} mice, in which no IgE or IgG was detected, anti-CD4 treatment resulted in complete abrogation of anaphylaxis. CD4-depleted CD4⁺ T-cell specific IL-4R α ^{-/-} mice, which had IgE antibody, experienced a transient temperature drop which soon returned to normal. This temperature drop was reduced by anti-CD4 treatment. However, even control treated mice of this strain did not experience reactions of the same grade as mice of the other strains, as illustrated by the symptom score graph.

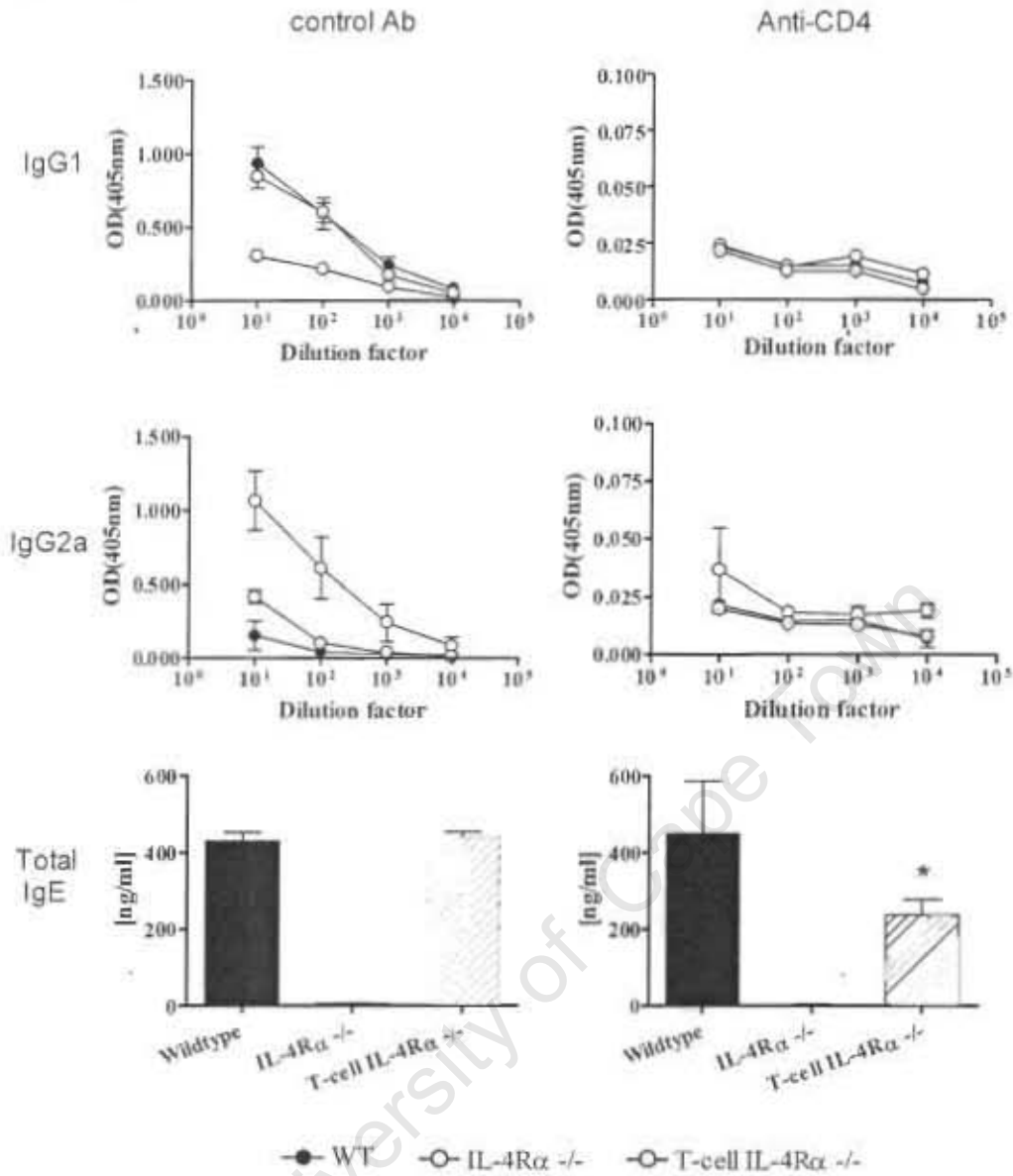


Figure 3.4.11. Depletion of CD4⁺ cells abrogated IgG production but not total IgE production. *Anisakis*-specific antibodies were measured in serum taken on the day of challenge. Data presented as mean \pm SEM. Statistical significance between wildtype and knockout mouse strains is shown. *, $P < 0.05$.

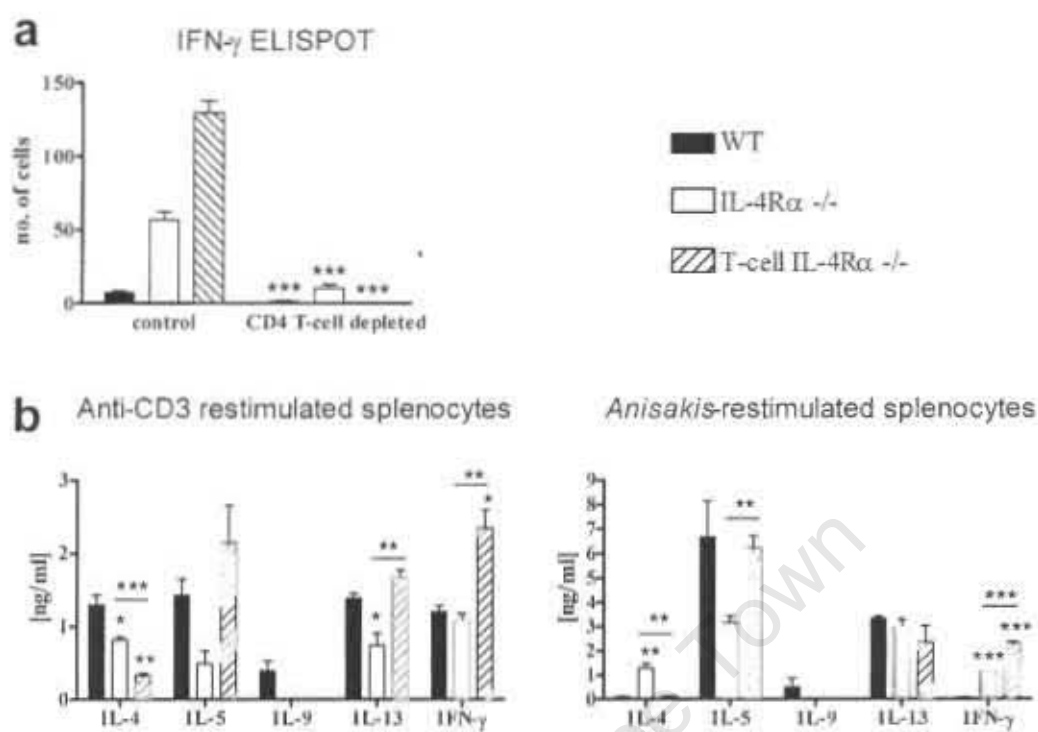


Figure 3.4.12. IFN- γ producing cells and cytokine production by restimulated splenocytes in anti-CD4 treated mice. a) ELISPOTS were performed to compare numbers of anti-IFN- γ producing splenocytes in control antibody and anti-CD4 treated mice. Control antibody treated groups were compared with anti-CD4 treated groups. b) Restimulated splenocytes from control antibody treated mice showed the same pattern of cytokine production as in previous experiments. Statistical significance between wildtype and knockout mouse strains mice is shown. Data presented as mean \pm SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

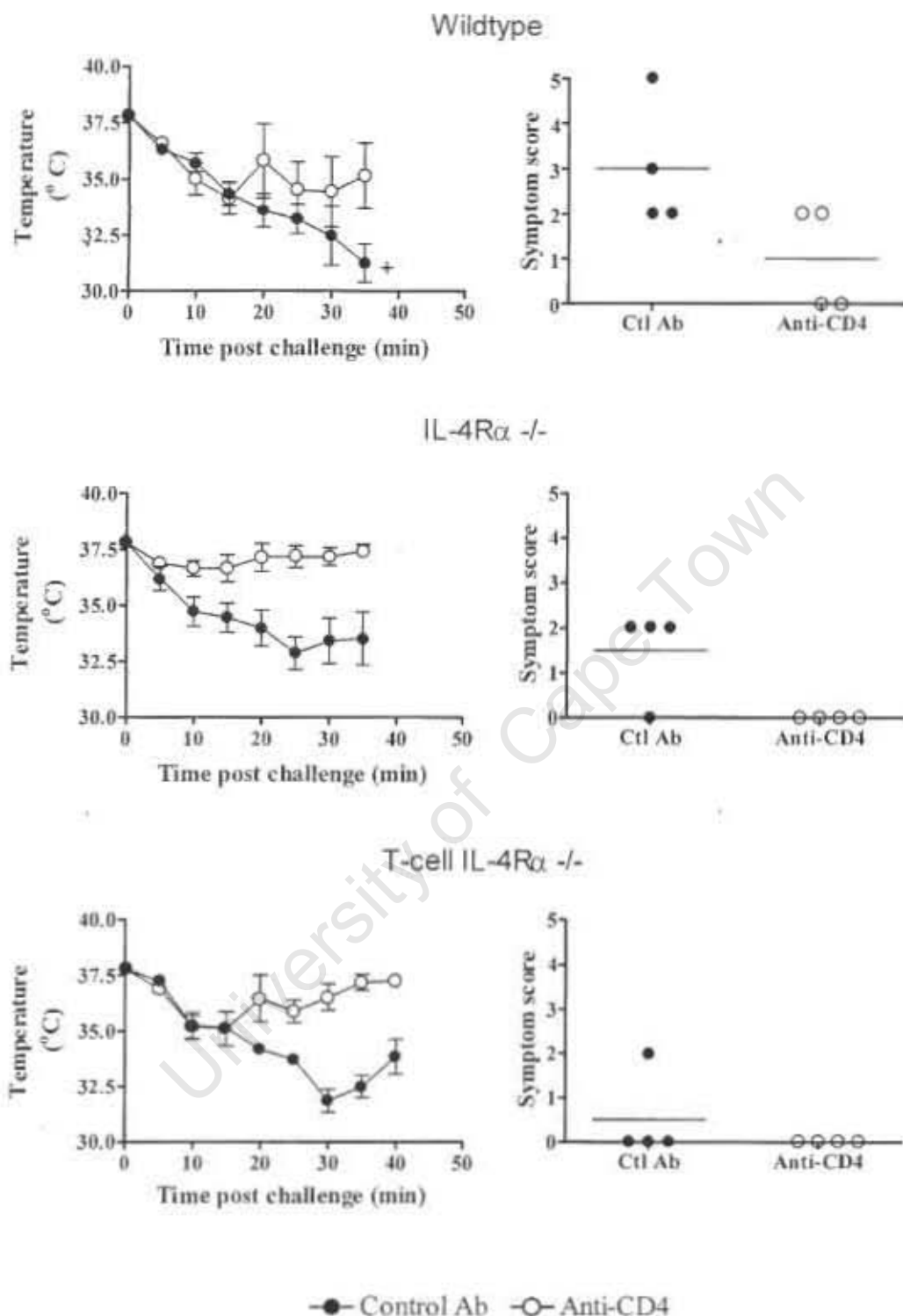


Figure 3.4.13. Hypothermia was reduced in wildtype, IL-4R α -/- and CD4⁺ T-cell specific IL-4R α -/- mice treated with anti-CD4. Mice were treated at days 0 and 14 with control antibody or anti-CD4. Temperatures were measured post challenge and reactions were scored as described in the methods. +, a mouse death.

3.4.6 IFN- γ depletion abrogates protection against anaphylaxis in CD4⁺ T-cell specific IL-4R α -/- mice

As the previous experiments demonstrated that T-cell specific IL-4R α knockout mice possessed high numbers of IFN- γ producing cells (Fig. 3.4.9 and Fig 3.4.12), and restimulated splenocytes produced high levels of IFN- γ (Fig. 3.4.10 and Fig. 3.4.12), it was hypothesized that IFN- γ was responsible for abrogation of anaphylaxis in this strain. IFN- γ depletion studies were therefore performed on wildtype, IL-4R α -/- and CD4⁺ T-cell specific IL-4R α -/- mice, using the OVA-induced anaphylaxis model. Following intravenous OVA challenge, wildtype mice treated with anti-IFN- γ experienced similar temperature loss to control treated mice but had slightly increased mortality (Fig. 3.4.14). In contrast, CD4⁺ T-cell specific IL-4R α -/- treated with control antibody had virtually no loss in temperature, but upon IFN- γ depletion these mice experienced rapid hypothermia and death. Scoring of anaphylactic reactions illustrated a significant increase in severity in IFN- γ depleted CD4⁺ T-cell specific IL-4R α -/- mice (Fig. 3.4.14). In a different representation of these data, Fig. 3.4.15 illustrates how anti-IFN- γ treatment removed differences in temperature loss between strains. However death still did not occur in any IL-4R α -/- mice, probably because of their inability to respond to IL-4 and IL-13, which are known to exacerbate anaphylaxis.

MMCP-1 levels in serum, a marker of systemic mast cell degranulation, were initially 6 times lower in control-treated CD4⁺ T-cell specific IL-4R α -/- mice than in both control and anti-IFN- γ treated wildtype mice (Fig. 3.4.16). After IFN- γ depletion, levels of MMCP-1 rose to the equivalent of wildtype levels, indicating an effect of IFN- γ in inhibiting mast cell degranulation.

In addition to having exacerbated systemic shock, IFN- γ depleted CD4⁺ T-cell specific IL-4R α -/- mice showed the intestinal swelling and hyperaemia characteristic of wildtypes.

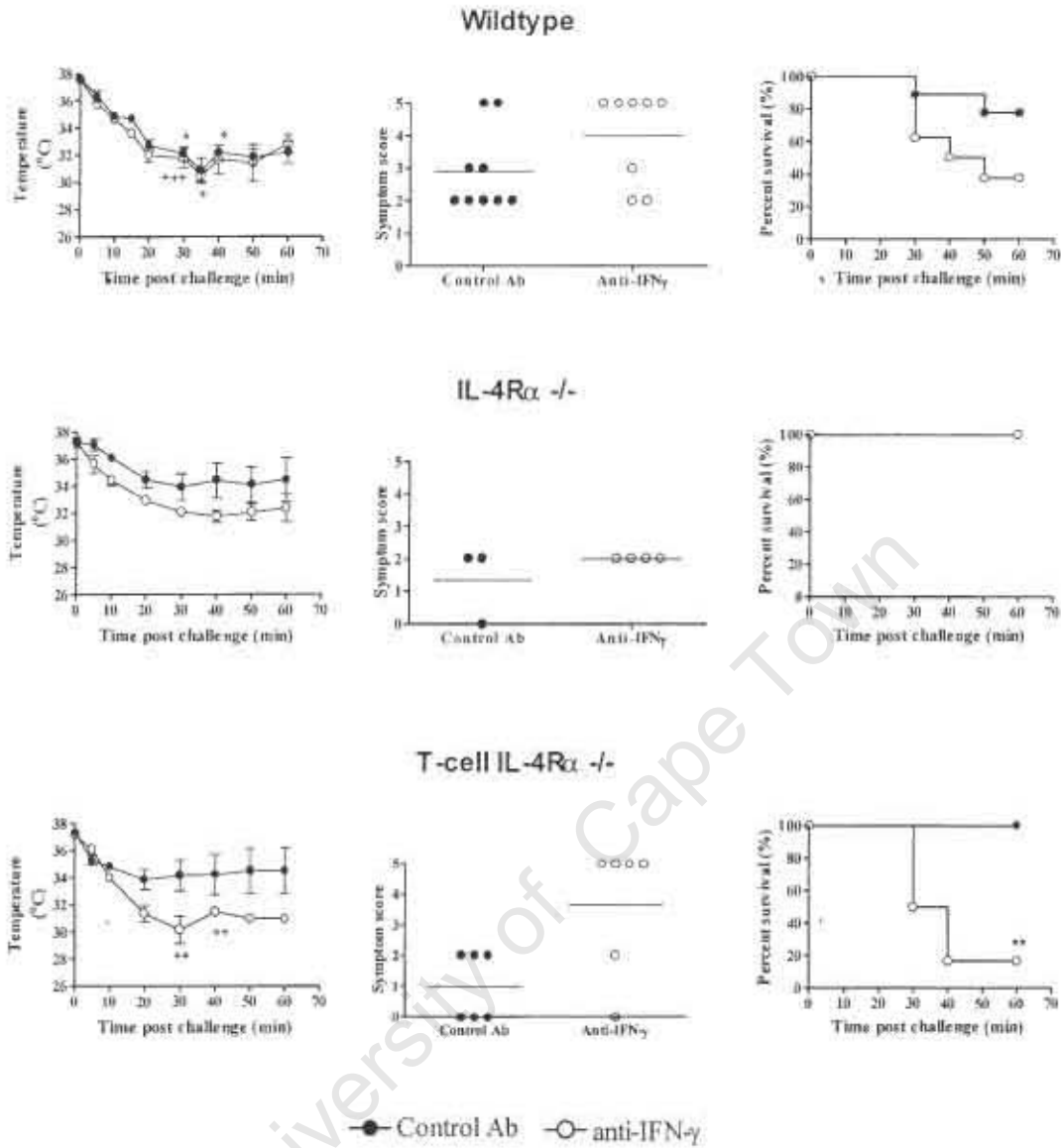


Figure 3.4.14. Anti-IFN- γ abrogates protection against anaphylaxis in CD4⁺ T-cell specific IL-4R α -/- mice. Temperatures were measured post challenge (left hand column) and anaphylactic reactions were scored (middle column). Survival curves appear in the right hand column. Data presented as mean +/- SEM. * P<0.05, ** P<0.01, +, a mouse death. Data representative of 2 experiments.

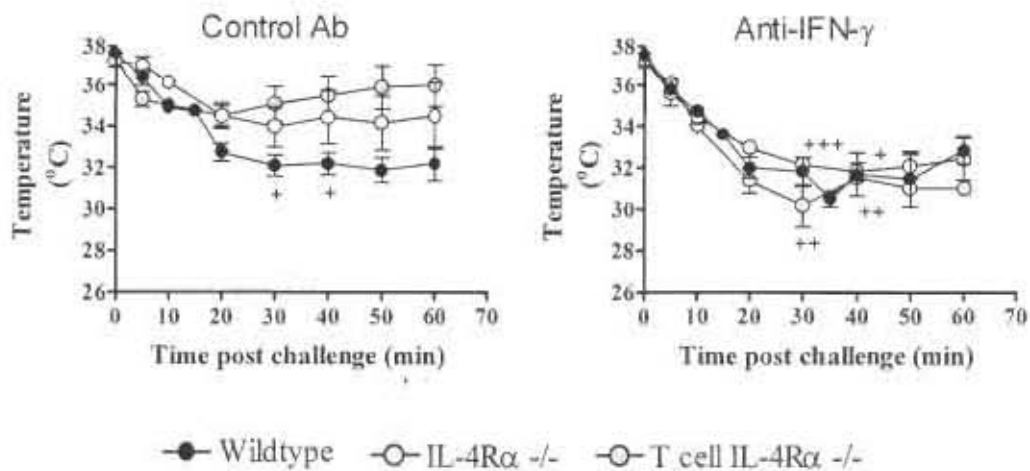


Figure 3.4.15. Anti-IFN- γ treatment removes differences in temperature loss between strains. This representation of the data, comparing control treated and anti-IFN- γ treated mice, shows how the differences in temperature loss between the strains are abrogated by anti-IFN- γ treatment. Data presented as mean \pm SEM. +, a mouse death.

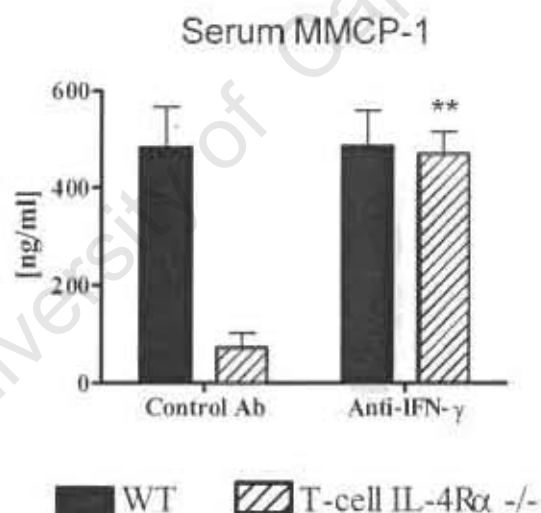


Figure 3.4.16. Anti-IFN- γ treatment is associated with elevated serum MMCP-1. MMCP-1 levels were measured in serum by ELISA, and levels in control-treated mice were compared statistically with levels in anti-IFN- γ treated mice. Data presented as mean \pm SEM. Statistical significance between control and anti-IFN- γ treated mice is shown. **, $P < 0.01$.

Analysis of cytokines produced by splenocytes restimulated with anti-CD3 was undertaken to determine whether anti-IFN- γ treatment, even at a late stage, could alter Th1/Th2 profiles. (Fig. 3.4.17) Levels of Th2 cytokines and IFN- γ produced by control antibody and anti-IFN-g treated splenocytes were not significantly different in either wildtype and CD4⁺ T cell specific IL-4R α -/- mice.

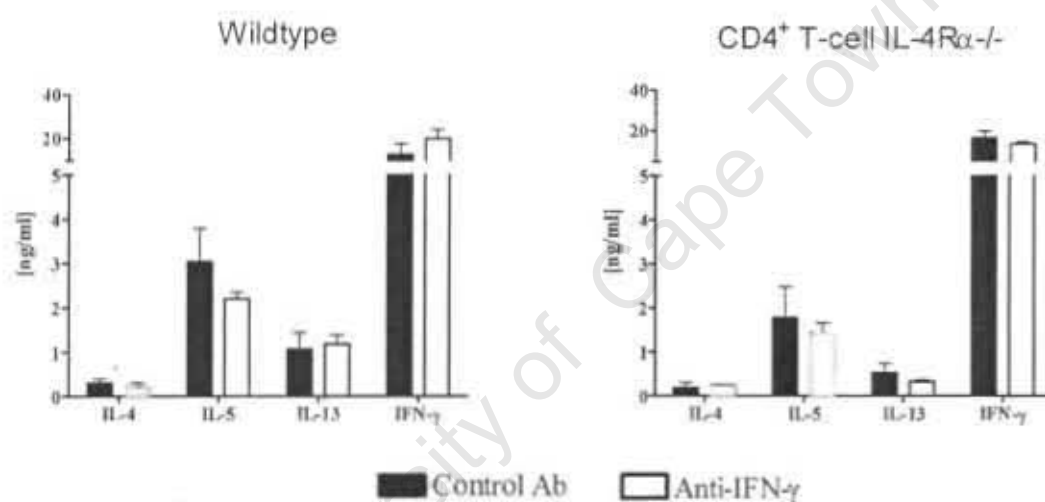


Figure 3.4.17. Th1/Th2 cytokines produced by restimulated splenocytes are not altered in anti-IFN- γ treated mice. IL-10 levels were increased in anti-IFN- γ treated T-cell specific IL-4R α -/- mice, but did not reach wildtype levels. Data presented as mean \pm SEM.

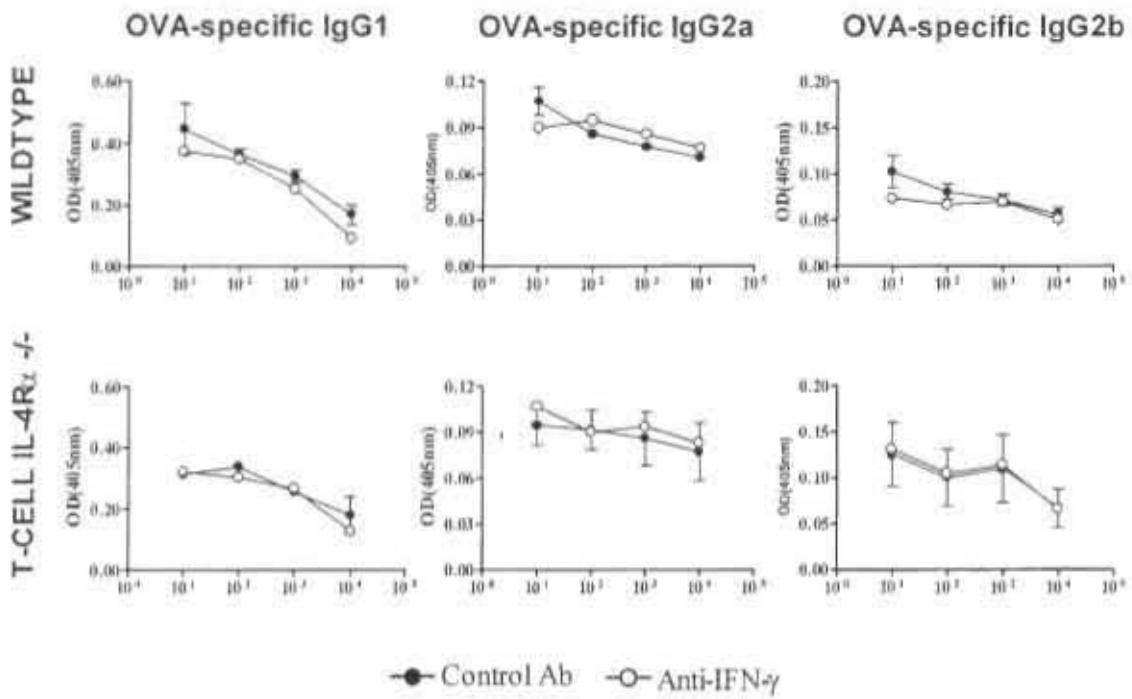


Figure 3.4.18. IgG antibody levels are not significantly altered by IFN- γ depletion. IgG antibodies were measured in serum taken after challenge in control-antibody treated and anti-IFN- γ treated mice. Data presented as mean \pm SEM.

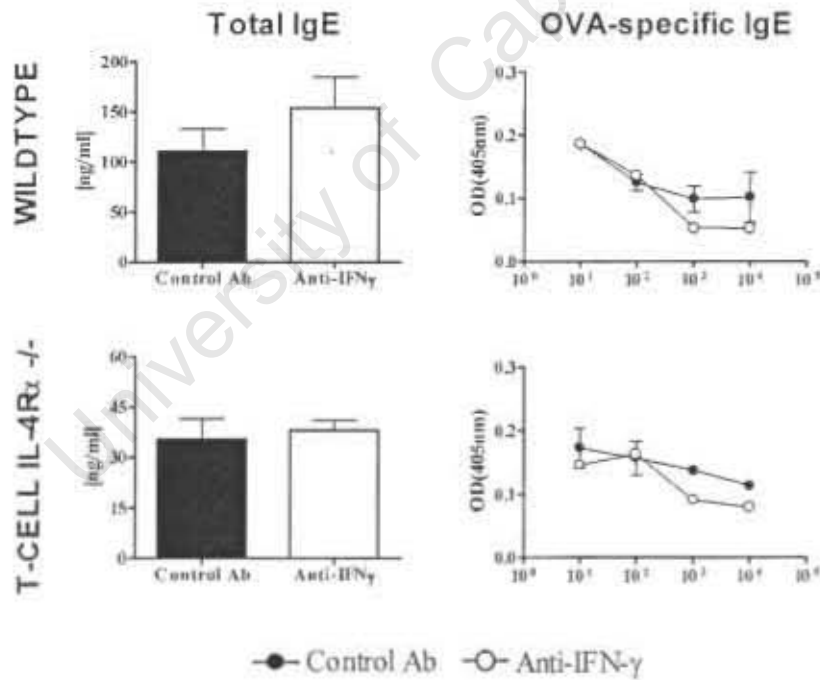


Figure 3.4.19. Total IgE and specific IgE levels are not significantly altered by IFN- γ depletion. Total and specific IgE levels were measured by ELISA in serum taken after intravenous challenge. Data presented as mean \pm SEM.

Antibody ELISAs did not show any significant difference in production of type 2 or type 1 antibodies between control-antibody and anti-IFN- γ treated mice (Fig. 3.4.18; Fig. 3.4.19). However, the levels of specific antibody detected were very low. ELISAs for the detection of specific antibodies in this model often showed only low antibody levels because the intravenous antigen challenge bound to serum antibodies, resulting in a reduction in free antibody available for detection (Fig.3.4.20). Attempting to bleed mice one day prior to the challenge resulted in an alteration of the severity of anaphylaxis (temperature drop and symptoms) due to the mice having decreased starting blood volume. Therefore, in order to obtain more reliable antibody results it would be necessary to conduct an experiment with sensitization and depletions but no antigen challenge specifically for the purpose of obtaining serum. However, specific IgG1 levels followed a titration curve in both strains, and total IgE was not affected, so the results strongly suggest that anti-IFN- γ treatment did not affect antibody levels.

3.4.7 Kinetics of OVA-alum sensitization

Previous experiments found an important protective effect of deletion of IL-4R α on CD4⁺ T-cells in an IFN- γ dependent mechanism in a model of anaphylaxis using two different antigens. In order to put these data into context, an investigation of the mechanisms of anaphylaxis in this model was needed. As the previous experiments demonstrated that OVA and *Anisakis* behave in the same manner in this model, it was decided that the model allergen OVA would be used in subsequent experiments to investigate mechanisms of anaphylaxis. As a protein, OVA is more easily available and has a known composition, whereas *Anisakis* extract is produced in limiting quantities and contains many different antigens that have not been characterized in detail. OVA is the standard allergen used in the literature, which would allow us to compare results obtained in our laboratory with results obtained in other laboratories.

The antibody and cytokine responses of wildtype mice sensitized with OVA adsorbed to alum according to the established protocol (Fig.3.4.1) were followed over 21 days. Samples were taken at day 1, 3 and 10 after the initial sensitization, as well as at day 15 (one day after the day 14 boost) and at day 21, the challenge time-point in the other

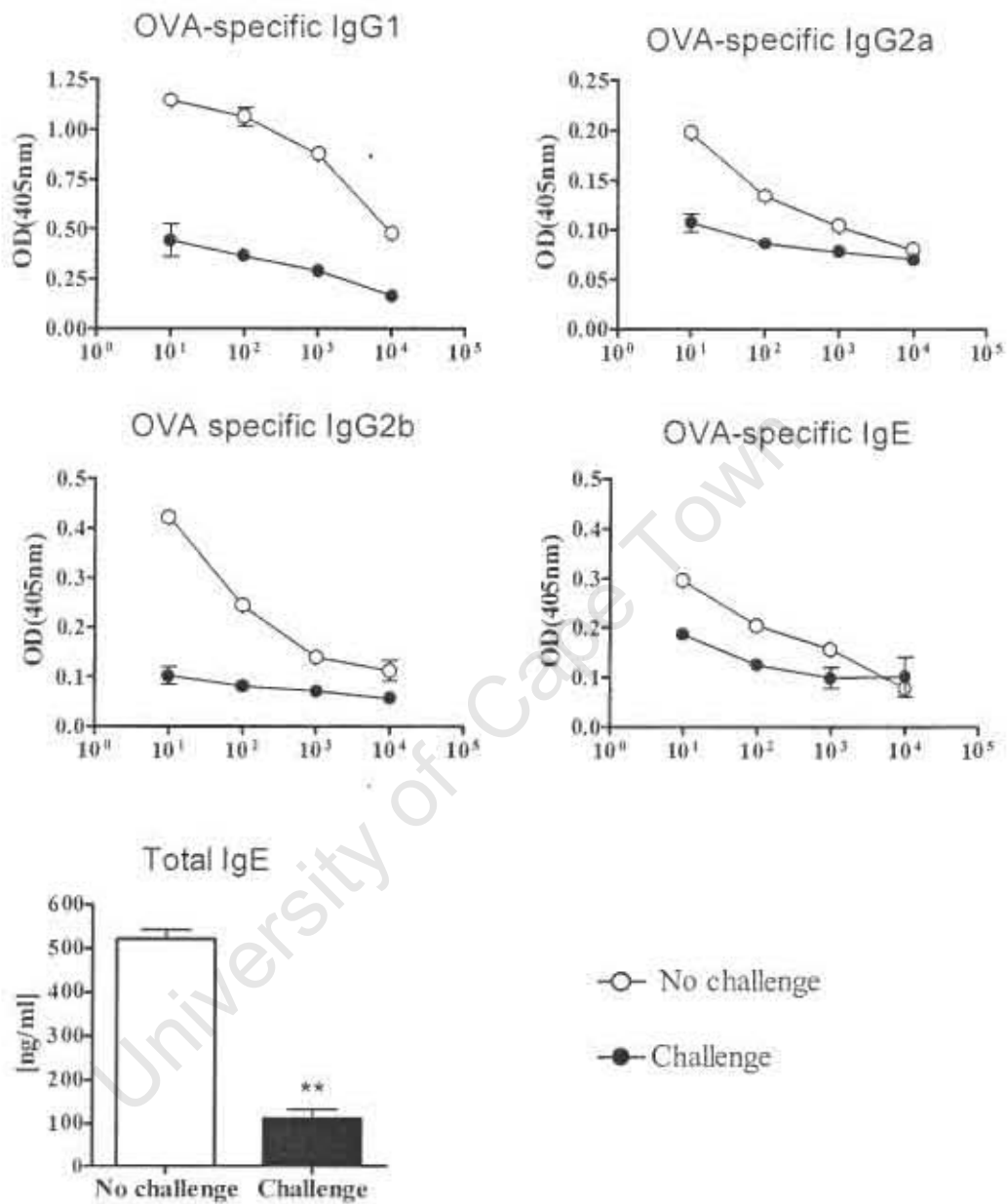


Figure 3.4.20. Intravenous antigen challenge causes a decrease in the amount of serum antibody measurable by ELISA. Antibody levels measured in challenged mice were substantially lower than antibodies measured in unchallenged mice which had been sensitized in parallel. This can be attributed to binding of free antibody by intravenously administered antigen. Data presented as mean +/- SEM. **, P<0.01.

experiments. PBS-alum sensitized mice were used as controls.

The results showed a predominant type 2 response, with high levels of IgG1, very low levels of IgG2a, and undetectable IgG2b (Fig. 3.4.21). OVA-specific IgG1 began to rise between day 3 and 10, then levelled off until the boost at day 14, after which it increased again. Small amounts of OVA-specific IgG2a also began to appear after the boost. Total IgE also began to rise within 10 days after sensitization, and remained fairly stable around the boost at day 14.

The kinetics of cytokine production from anti-CD3 restimulated splenocytes indicated that production of both IL4 and IL-5 increased after the initial sensitization (by day 3), were decreased again by the time of the boost, and increased again after the boost (Fig. 3.4.22). Splenocytes taken at day 21 produced significantly higher levels of both IL-4 and IL-5 levels than splenocytes taken from day 1, or from PBS-alum sensitized mice. A similar trend towards increased IL-13 and IL-9 was observed, while IFN- γ production remained fairly constant throughout the sensitization regime. Interestingly, after the boost, levels of IL-10 and TGF- β were also raised. It is possible that repeated sensitization would lead to a regulatory response and immunological tolerance. Though at day 21 Th2 cytokines were still raised, sampling at more time-points before and after day 21 would show whether Th2 levels were still on the increase or beginning to decrease by day 21 due to the rise in regulatory cytokines.

3.4.8 IL-4 depletion during sensitization reduces the severity of anaphylaxis in wild-type mice

Anti-IL-4 treatment during the sensitization phase in wildtype mice led to a significant decrease in hypothermia and severity of anaphylaxis (Fig. 3.4.23). This correlated with a decrease in IL-5, IL-9 and IL-13 and an increase in the amounts of IL-10 and TGF- β produced by restimulated splenocytes (Fig.3.4.24). In addition, levels of the type 1 antibody IgG2a were increased after IL-4 depletion, while total IgE was decreased (Fig.3.4.24). IgG1 levels were unaffected, and IgG2b levels were low in both groups.

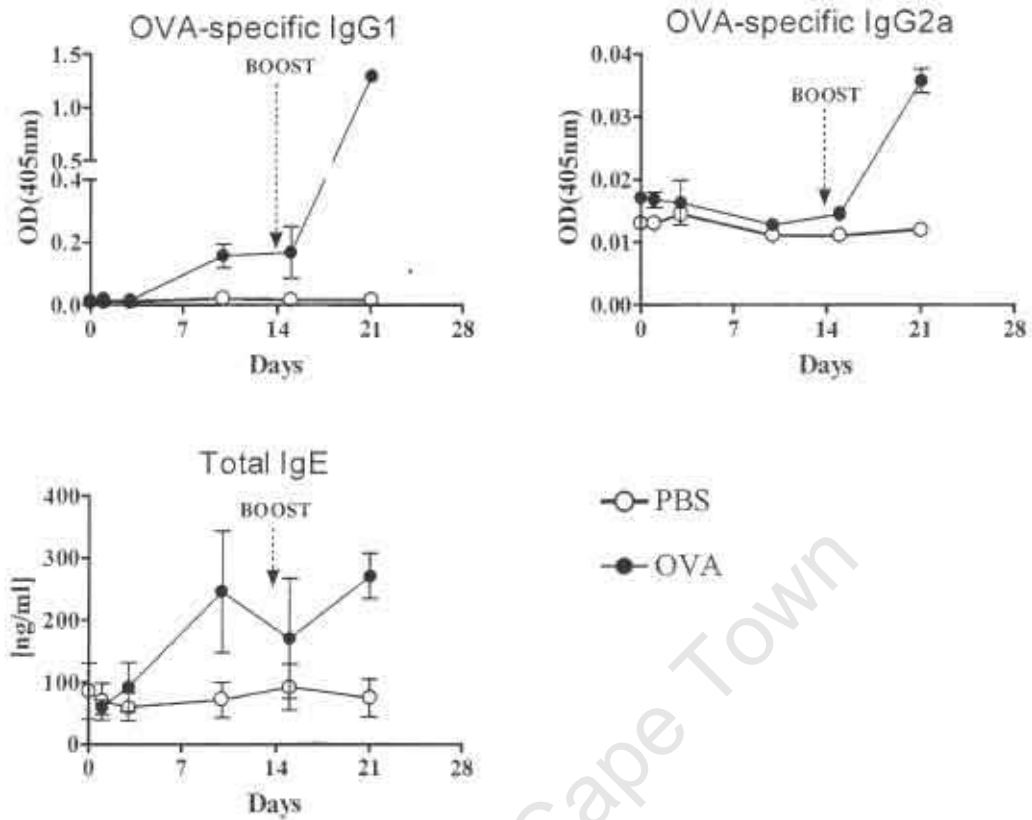


Figure 3.4.21. Antibody kinetics during intraperitoneal sensitization. Mice were sensitized intraperitoneally at day 0 with alum-adsorbed OVA, and again at day 14. Antibody production over time was measured by ELISA. Data presented as mean \pm SEM.

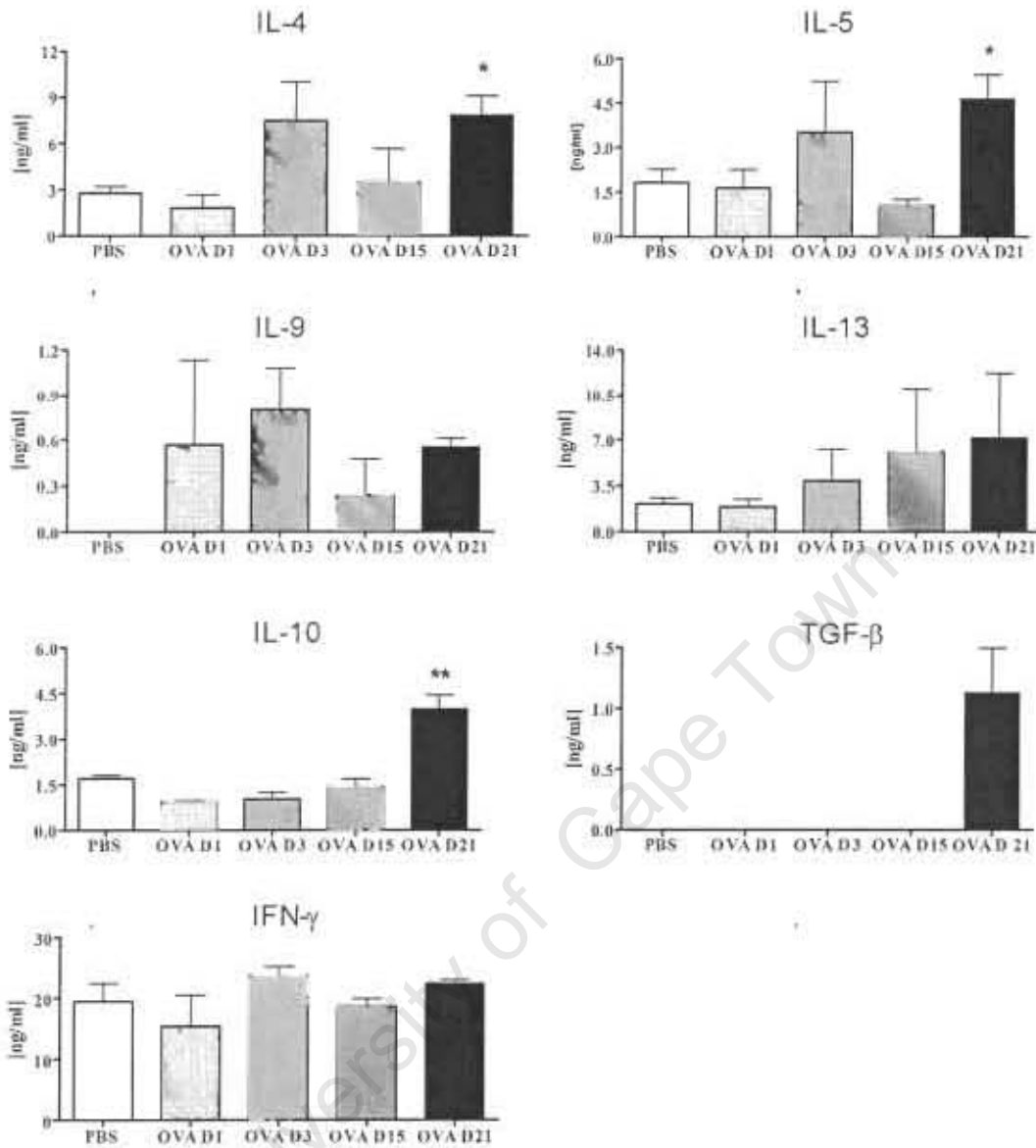


Figure 3.4.22. Cytokine production by restimulated splenocytes at different time points during intraperitoneal sensitization. Mice were sensitized intraperitoneally at day 0 with alum-adsorbed OVA, and again at day 14. Cytokine production was measured from restimulated splenocytes taken at various points in the sensitization schedule. Cytokine levels were statistically evaluated against cytokine levels produced by PBS-sensitized mice. Data presented as mean \pm SEM. Statistical significance was calculated against PBS-sensitized mice. *, $P < 0.05$.

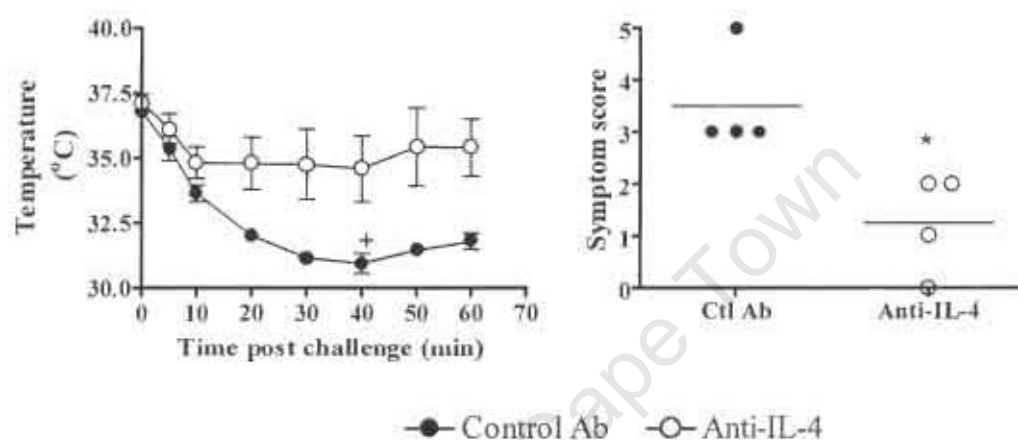


Figure 3.4.23. IL-4 depletion during sensitization reduces the severity of OVA-induced anaphylaxis. Mice were sensitized at day 0 and day 14 with alum-adsorbed OVA, and treated at day 0 and day 14 with anti-IL-4 or a control antibody. Intravenous challenge with OVA was performed at day 21. Rectal temperatures were measured and anaphylactic reactions were scored (see Methods). Data presented as mean \pm SEM. *, $P < 0.05$; +, a mouse death.

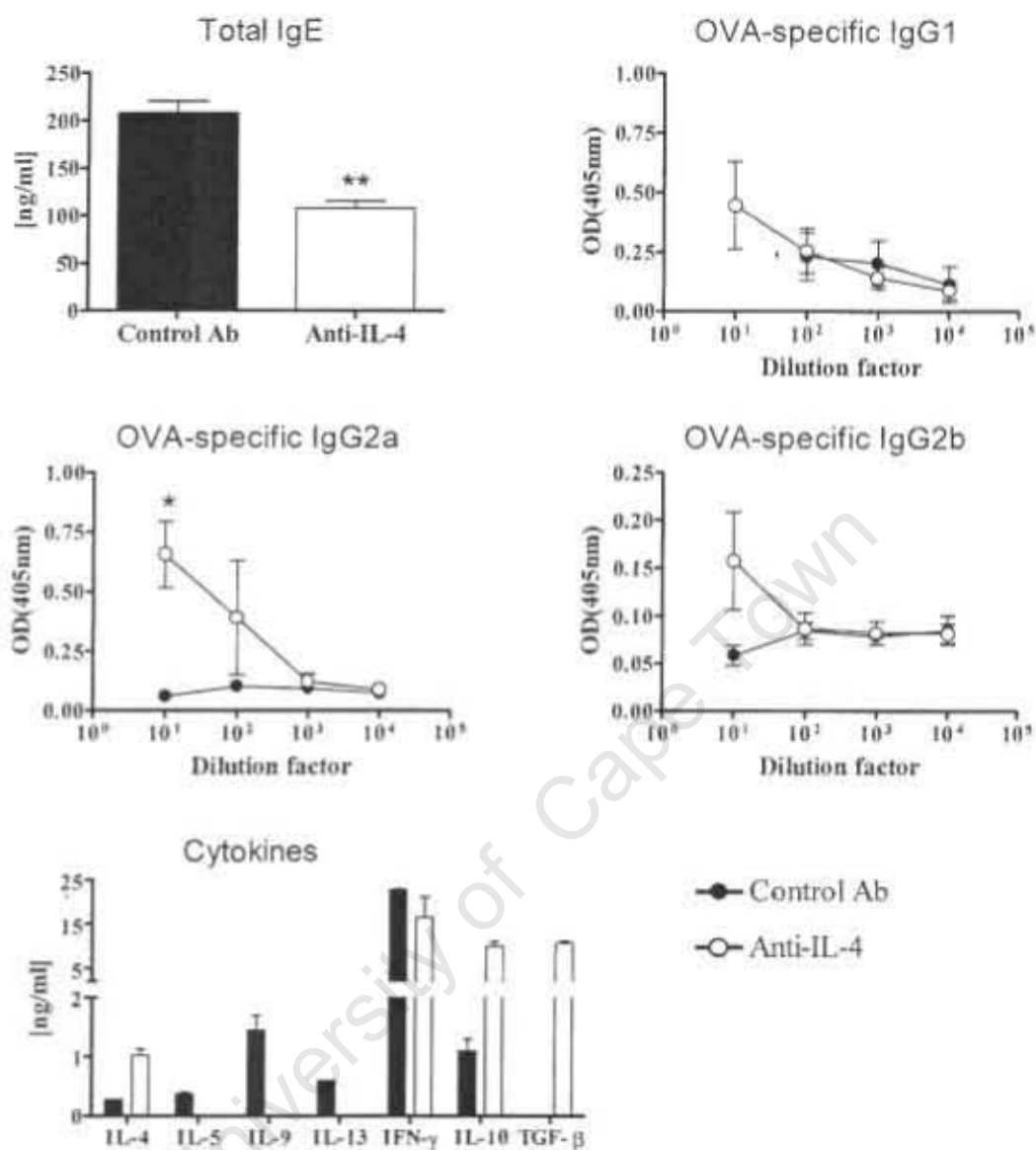


Figure 3.4.24. Anti-IL-4 treatment during sensitization affects antibody levels and cytokine production by restimulated splenocytes. Mice were sensitized at day 0 and day 14 with alum-adsorbed OVA, and treated at day 0 and day 14 with anti-IL-4 or a control antibody. Antibodies were measured in serum taken at day 21 after intravenous challenge with OVA, and cytokine production was measured from restimulated splenocytes taken at day 21. Data presented as mean \pm SEM. Statistical significance between control and anti-IL-4 treated mice is shown. *, $P < 0.05$; **, $P < 0.01$.

3.4.9 OVA-induced anaphylaxis is abrogated by Fc γ RII/III depletion

Some models of systemic anaphylaxis in mice are mediated primarily by IgG and the Fc γ RII/III^{23, 24}, while others are IgE-mediated^{27, 51}. To determine whether anaphylaxis in our model was IgG- or IgE-mediated, we performed depletion studies using anti-Fc γ RII/III, anti-IgE or a control rat IgG antibody. Fig.3.4.25 shows that anti-IgE treatment effectively depleted IgE but did not significantly alter IgG levels. Anti-Fc γ RII/III treatment did not affect IgG levels, but inexplicably seemed to reduce IgE levels. However the anti-IgE group could act as a control for any effects due to depletion of IgE and not blocking of Fc γ RII/III in this group.

Following intravenous challenge, rectal temperatures dropped in both the control group and the anti-IgE treated group, but did not drop in the anti-Fc γ RII/III treated group (Fig. 3.4.26). Furthermore, signs of anaphylaxis were almost completely abrogated in anti-Fc γ RII/III treated mice, with only 2/9 mice having mild reactions in pooled data from two experiments (Fig. 3.4.26). In contrast, anti-IgE treated mice had similar mortality rates and anaphylactic scores to controls. Mouse mast-cell protease-1 was measured in the gut and serum as a measure of mast cell degranulation (Fig. 3.4.27). Depletion of Fc γ RII/III correlated with a strong decrease in mast cell degranulation in the gut as compared to control antibody and anti-IgE treated mice, though serum MMCP-1 was decreased in both the anti-Fc γ RII/III and the anti-IgE treated mice. The decreased MMCP-1 levels in anti-Fc γ RII/III treated mice may be attributed to lowered IgE levels (Fig. 3.4.26). It should be noted however that the lower levels of MMCP-1 measured in the gut of Fc γ RII/III may also be a result of the lack of intestinal hyperaemia in this group.

3.4.10 Anaphylaxis is not significantly affected by the absence of IL-4R α on macrophages/neutrophils

In previous publications, two research groups found a role for macrophages in IgG-mediated anaphylaxis^{23, 24}. It is known that macrophages can be polarized into two different metabolic states depending on whether they are in a type 1 or type 2 environment⁴⁶. Both IL-4 and IL-13, via IL-4R α , have been shown to promote the

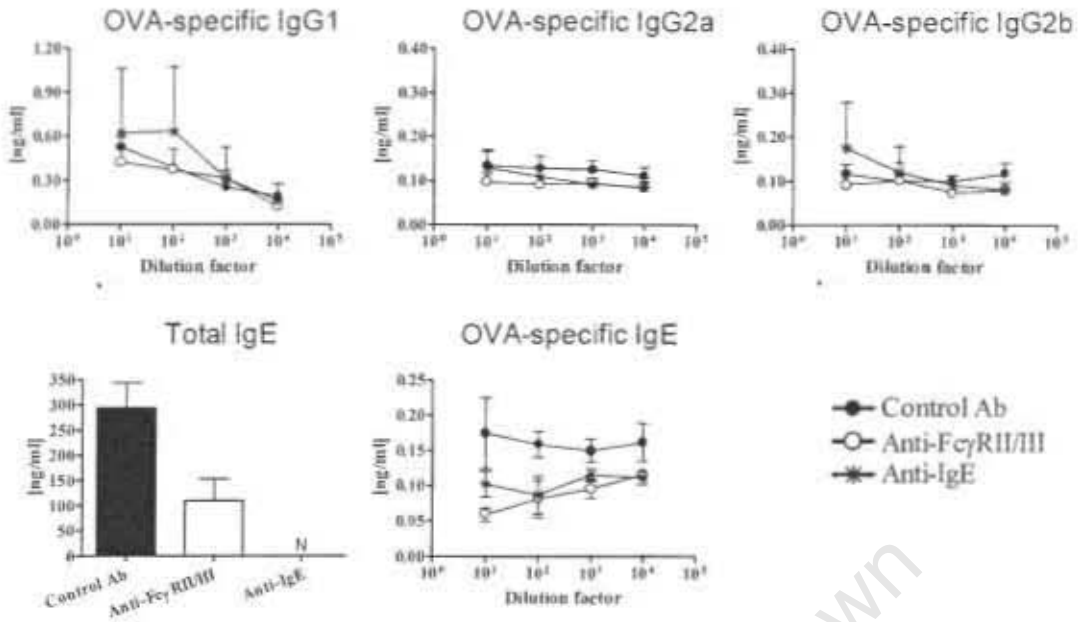


Figure 3.4.25. The effect of anti-IgE and anti-FcγRII/III on serum antibody levels. Mice were sensitized at day 0 and day 14 with alum-adsorbed OVA, and treated at day 18 and day 20 with anti-IgE, anti-FcγRII/III or a control antibody. Antibody levels were measured in serum taken after intravenous challenge. Data presented as mean +/- SEM. N, undetectable.

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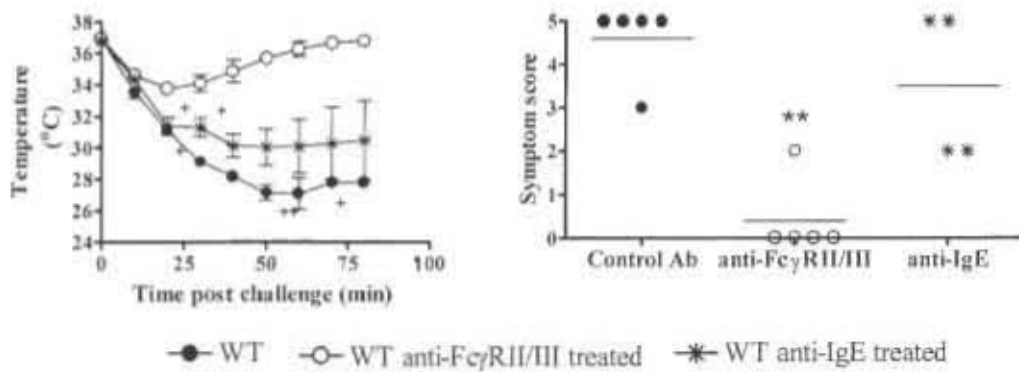


Figure 3.4.26 Anti-Fc γ RII/III treatment abrogates anaphylaxis. Mice were sensitized intraperitoneally at day 0 and day 14 with alum-adsorbed OVA, and challenged intravenously at day 21 with OVA. Temperatures were measured by rectal thermometer and reactions were scored as described in the Methods.

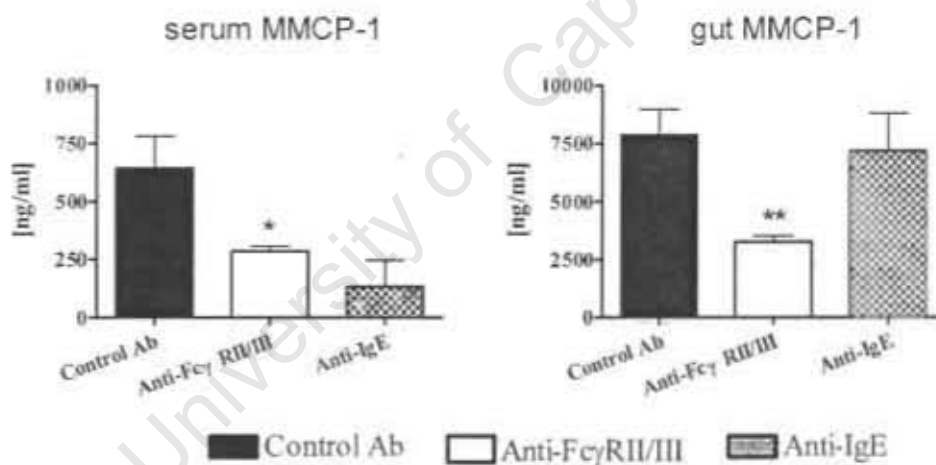


Figure 3.4.27. The effects of anti-IgE and anti-Fc γ RII/III treatment on serum and gut MMCP-1 levels. MMCP-1 levels were measured in serum taken after challenge and in gut homogenates. Data presented as mean \pm SEM. Statistical significance between mice treated with control antibody and those treated with neutralizing antibody is shown. *, $P < 0.05$; **, $P < 0.01$.

phenotype known as the alternatively activated macrophage. In order to determine whether alternatively activated macrophages play a role in anaphylaxis, experiments were performed using previously characterized cell specific knockouts selectively lacking the IL-4R α on macrophages and neutrophils (LysM^{Cre}IL-4R α ^{-lox} mice)⁴².

The antibody responses in macrophage/neutrophil specific IL-4R α ^{-/-} mice were virtually identical to those in wildtype mice (Fig. 3.4.28). Sensitization with OVA-alum resulted in high levels of IgG1 and raised total IgE. Restimulated splenocytes from this strain additionally produced a similar profile to restimulated splenocytes from wildtypes, although both IL-4 and IFN- γ were produced in higher amounts in splenocytes from macrophage/neutrophil specific IL-4R α ^{-/-} mice (Fig. 3.4.29). After intravenous challenge with OVA, macrophage/neutrophil specific IL-4R α ^{-/-} mice developed anaphylaxis to more or less the same extent as wildtype mice (Fig. 3.4.30). There was a non-significant trend towards a very slight decrease in hypothermia and symptom score in macrophage/neutrophil specific IL-4R α ^{-/-} mice (Fig. 3.4.30), accompanied by a significant decrease in MMCP-1 levels in the serum (Fig. 3.4.31). However, as macrophage/neutrophil specific IL-4R α ^{-/-} mice still underwent severe and often fatal anaphylaxis, overall it could be concluded that IL-4R α on macrophages and neutrophils, and by inference the presence of alternative macrophages, do not play a significant role in this model of anaphylaxis.

3.4.11 The effect of mast cell depletion on anaphylaxis

Unfortunately the anti-ckit treatment did not completely deplete mast cells, and there was not enough antibody to repeat the experiment. However, there was a trend towards decreased MMCP-1 levels in serum with increasing anti-ckit injections (Fig. 3.4.32), indicating that partial depletion was obtained. Interestingly, mice in groups receiving 2 or 3 anti-ckit injections had significantly increased survival rates and reduced symptom scores compared to mice which had been given 0 or 1 injections (Fig.3.4.33). In contrast, both groups of mice experienced similar temperature loss. These results may indicate that mast cells play a role in increasing the severity of anaphylaxis and risk of death in this model. However no conclusions can be drawn without repeating the experiment.

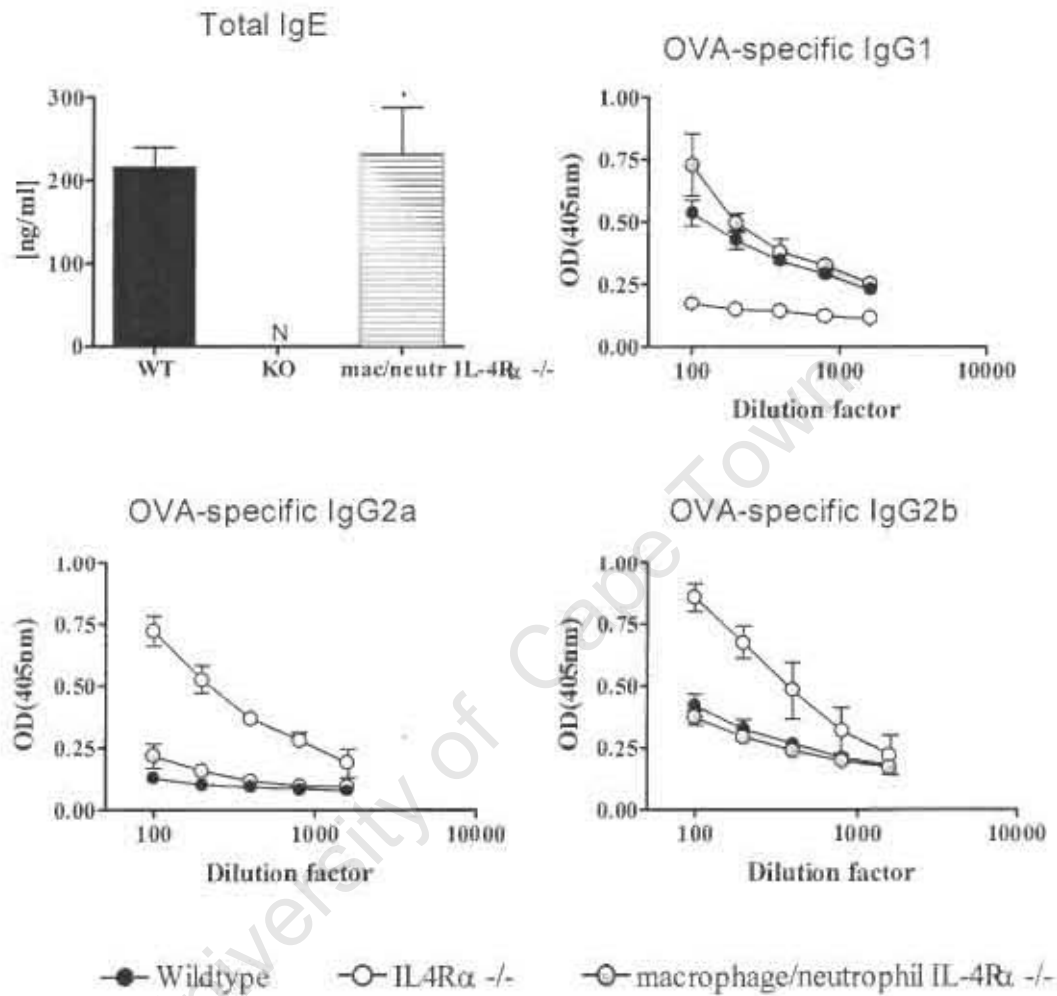


Figure 3.4.28. Antibody production in macrophage/neutrophil specific IL-4R α -/- mice. Mice were sensitized intraperitoneally at day 0 and 14 with alum-adsorbed OVA and challenged intravenously at day 21. Antibody levels were measured by ELISA. N, undetectable. Data presented as mean +/- SEM.

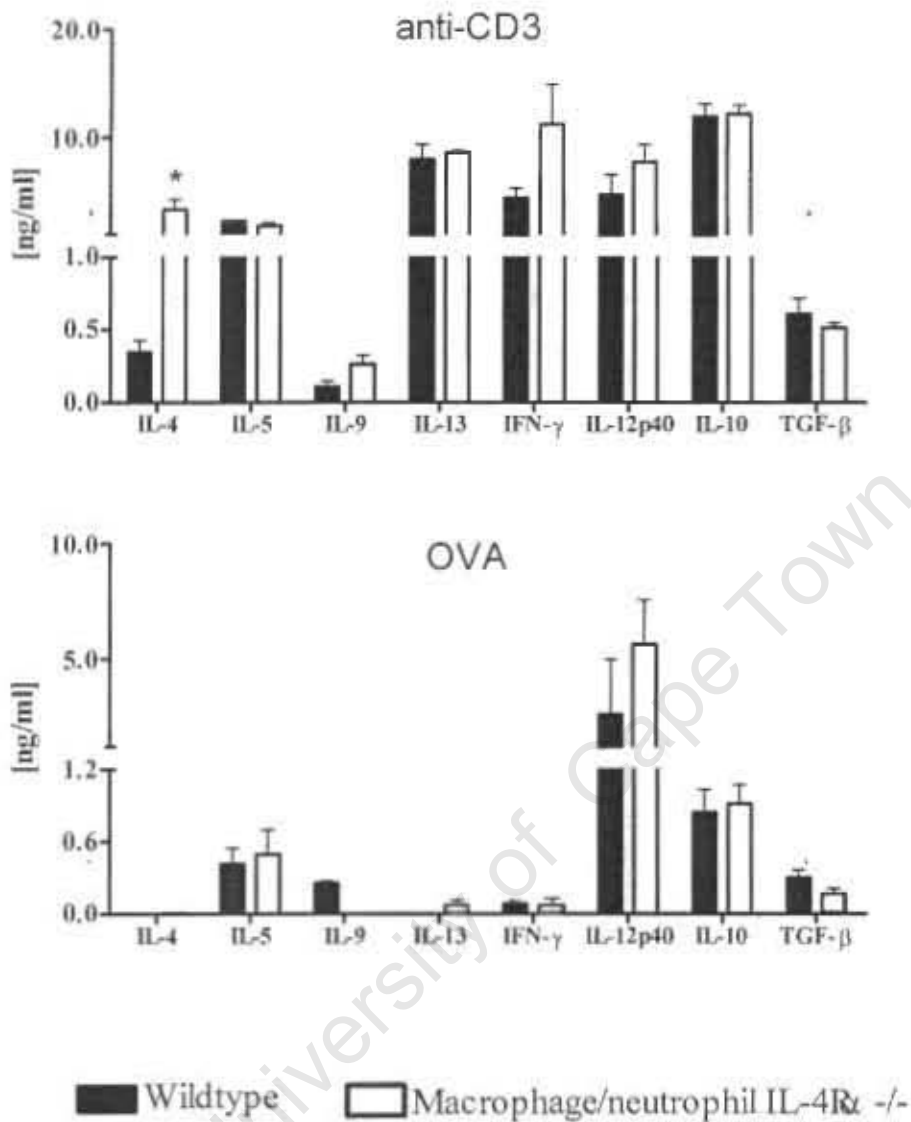


Figure 3.4.29. Cytokine production by restimulated splenocytes in wildtype and macrophage/neutrophil specific IL-4R α -/- mice. Mice were sensitized intraperitoneally with alum-adsorbed OVA at day 0 and day 14, and challenged intravenously with OVA at day 21. Splenocytes taken after challenge were restimulated with anti-CD3 or OVA. Data presented as mean \pm SEM. Statistical significance between wildtype and knockout mice is shown. *, $P < 0.05$.

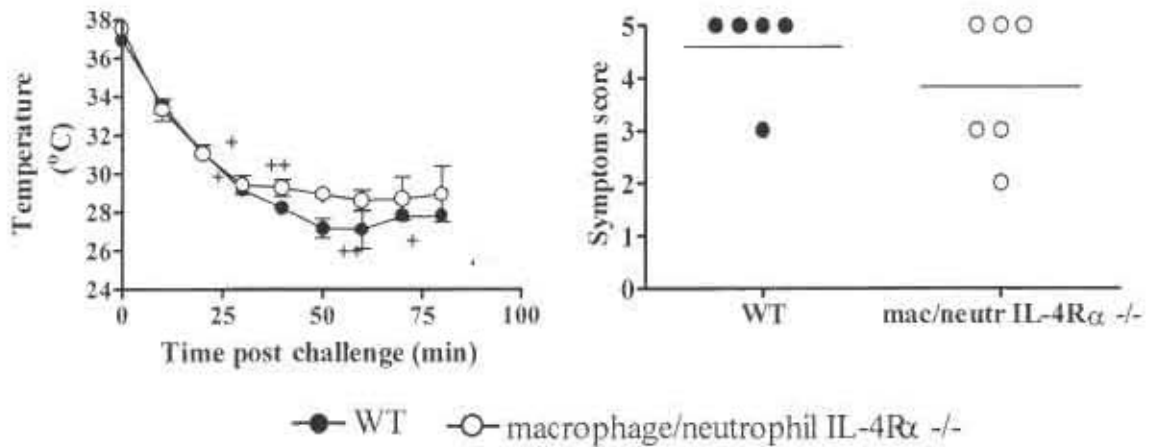


Figure 3.4.30. Macrophage/neutrophil specific deletion of IL-4R α does not significantly affect anaphylaxis. Mice were sensitized intraperitoneally with alum-adsorbed OVA at day 0 and day 14, and challenged intravenously with OVA at day 21. Temperatures were measured with a rectal thermometer and reactions were scored as described in the Methods. Data presented as mean \pm SEM. +, death of a mouse.

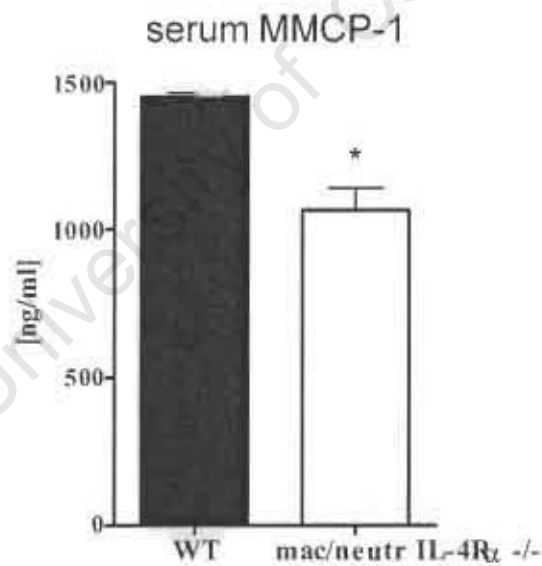


Figure 3.4.31. MMCP-1 levels in macrophage/neutrophil specific IL-4R α -/- mice. MMCP-1 levels were measured in serum after challenge. Data presented as mean \pm SEM. Statistical significance between wildtype and knockout mice is shown. *, P < 0.05

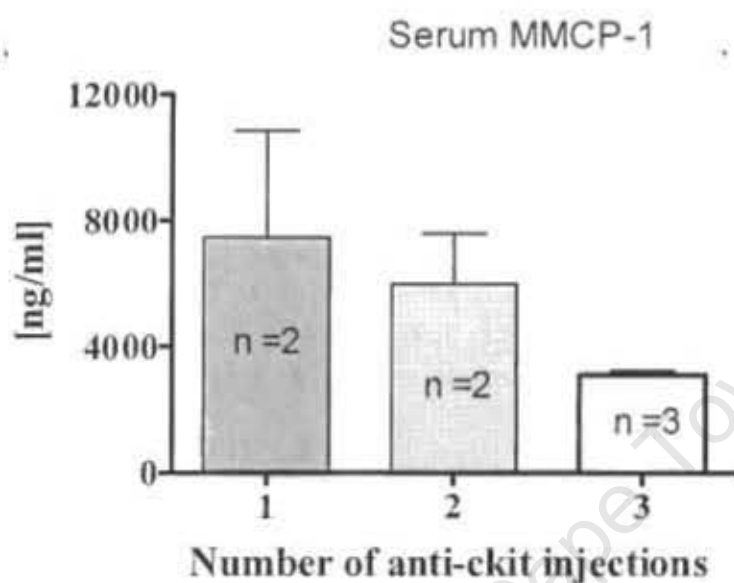


Figure 3.4.32. Serum MMCP-1 levels decreased with increasing anti-ckit injections. n = the number of serum samples obtained. It is often impossible to obtain blood from mice which have had severe anaphylaxis due to extensive vascular leakage causing a loss of blood volume. No serum was obtained from any of untreated mice, reflecting the increased severity of anaphylaxis in this group. Data presented as mean \pm SEM.

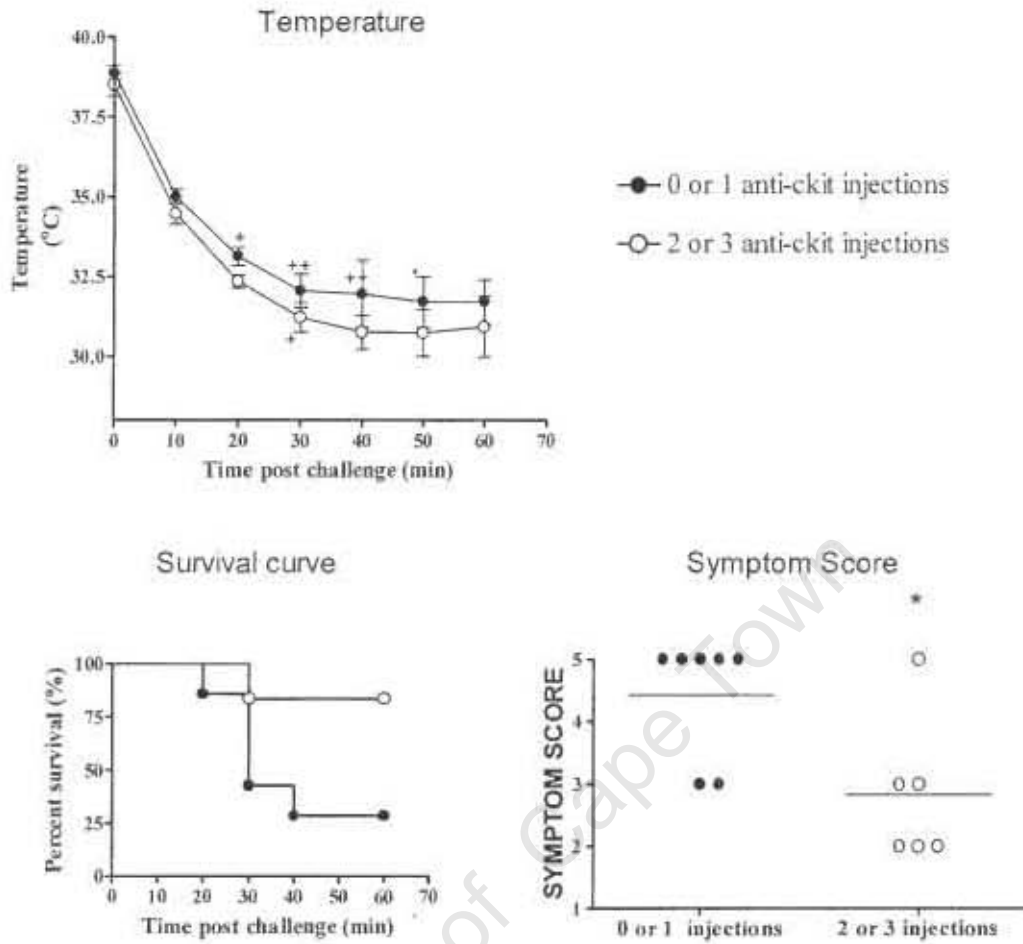


Figure 3.4.33. Partial mast cell depletion was associated with an increased survival rate and decreased symptom score. Mice were sensitized intraperitoneally at days 0 and 14 with alum-adsorbed OVA, treated with anti-ckit at days 18 and 20, and challenged with OVA at day 21. Temperatures were measured with a rectal thermometer and reactions were scored according to the system described in the Methods.

3.5 DISCUSSION

Understanding the immunological mechanisms surrounding anaphylaxis is essential for the design of better preventative and therapeutic strategies. Certain substances, such as peanuts, egg, seafood, *Hymenoptera* venom, antibiotics and latex, are more prone to elicit anaphylactic reactions than others. *Anisakis* has recently received much attention for its ability to cause anaphylaxis, but it is uncertain whether live larvae are required to elicit anaphylaxis or whether *Anisakis* proteins themselves are intrinsically allergenic. The data presented in this chapter show that *Anisakis* extract behaves similarly to the egg allergen OVA in a model of systemic anaphylaxis. Furthermore, this chapter explores mechanisms of anaphylaxis, using cell-specific IL-4R α deficient mice to show that IL-4R α expressed on CD4⁺ T cells but not macrophages and neutrophils is critical in mediating responses that lead to antigen-induced anaphylactic shock, and that IFN- γ plays a key role in protection against anaphylaxis.

In this model of antigen-induced anaphylaxis, *Anisakis* extract was able to elicit the same type of reactions as the model-allergen OVA. At equal doses, OVA elicited slightly more severe reactions, but this was likely due to the fact that it is a single allergen whereas *Anisakis* extract contains a wide variety of proteins, only some of which would be allergenic. Recently, a similar study was published in which *Anisakis*-induced anaphylactic reactions occurred following intravenous challenge of mice sensitized intraperitoneally with *Anisakis simplex* extract, pertussis toxin and alum⁵². The authors additionally challenged sensitized mice orally with *Anisakis* extract, but no reactions occurred through this route of exposure. This suggests that live infection with *Anisakis* larvae is more effective than *Anisakis* extract at sensitising for ingestion-related allergies in mice.

Despite the fact that live larvae appear to be stronger sensitizers than extract alone, the data presented here and in chapter two indicate that certain *Anisakis* proteins have allergenic properties, as opposed to the view that allergic reactions to *Anisakis* are based solely on an inflammatory response to a live helminth infection¹⁵. This is supported by the fact that the same anaphylaxis model was tested in our laboratory using pilchard

extract, but reactions did not occur after intravenous challenge⁵³. Furthermore, both human and dog hookworm can cause an eosinophilic, allergic-type response in the intestine, but neither causes systemic allergic reactions the way *Anisakis* does^{54,55}.

It has been proposed that proteolytic enzymes of parasitic worms are particularly effective at eliciting Th2 responses. Several important allergens from house dust mites, moulds, cats and fruits have protease activity that is thought to enhance their allergenicity and trigger sensitization to concomitantly administered antigens⁵⁶. *Anisakis* secretes proteolytic enzymes in order to burrow into the submucosa, some of which may be allergenic and enhance the allergenicity of other *Anisakis* proteins by allowing them access to subepithelial immune tissue. The allergenicity of certain *Anisakis* proteins suggests that reactions to *Anisakis* should be considered a genuine allergy, and not only a pseudo-allergic inflammatory response. This would be compatible with case reports of allergy due to airborne *Anisakis* proteins⁵⁷⁻⁵⁹. As previously suggested, chronic urticaria may be as a result of constant exposure to *Anisakis* proteins in food in sensitized individuals, and not only repeated infections with live larvae⁶⁰.

Subsequent to the establishment of a model of anaphylaxis, we aimed to elucidate the role of IL-4R α functioning in certain cell types. The IL-4R α plays an important role in several models of allergy by mediating signaling of IL-4 and IL-13⁶¹⁻⁶⁴. IL-4 and IL-13 signalling is important for creating the environment required for allergic responses, including B-cell IgE and IgG1 secretion, Th2 differentiation and associated accumulation of effector cells such as mast cells and eosinophils^{50,64,65}. Both IL-4 and IL-13 also exert effects upon epithelial, endothelial and smooth muscle cells by promoting chemokine production, vasodilation and contractility, and can exacerbate anaphylaxis by increasing cellular responsiveness to vasoactive mediators and shifting the balance of ion and fluid flow^{25,41,64,66-68}. IL-4, but not IL-13 increases intestinal mastocytosis, while IL-13 increases susceptibility to fatal anaphylaxis by contributing to lung pathology such as goblet cell hyperplasia, mucus hypersecretion, fibrosis and eosinophilia^{64,66}. Accordingly, the present study and others^{41,50} have shown that the IL-4R α plays an important role in anaphylaxis, with null mutant mice maintaining resistance to death and

intestinal pathology except at very high challenge doses. Unlike anti-FcγRII/III neutralization, the absence of IL-4Rα did not abrogate anaphylaxis entirely, but rather reduced the severity of anaphylaxis. This is consistent with a role for IL-4 and IL-13 in exacerbating anaphylaxis⁴¹. The cell specific requirements for IL-4Rα surface expression in anaphylaxis have not been previously investigated.

Alternative activation of macrophages is induced by IL-4 and IL-13 and is thought to play a role in chronic allergic disease⁴⁶. However, the role of alternatively activated macrophages in acute allergic diseases is unknown. The results of this study show that macrophage and neutrophil activation through the IL-4Rα is not essential for pathology and mortality in this model of anaphylaxis. This demonstrates that alternatively activated macrophages do not have an important role in this type of allergic reaction, if any. A previous model of anaphylaxis found that macrophage production of PAF was the major pathway for IgG-dependent murine anaphylaxis²⁴. While macrophages were not depleted in this study, the data here indicate that if macrophages do play a role in this model, it would have to be via classical activation by IL-12. The recently discovered role for NO in mediating the vasodilation associated with anaphylaxis would support this conjecture⁶⁹, as classically activated macrophages are an important source of NO⁴⁶.

Deletion of IL-4Rα from CD4⁺ T-cells alone abrogated or strongly reduced anaphylaxis, despite maintenance of a Th2 response and the presence of antigen-specific IgE and IgG. Experiments using different challenge doses demonstrated that protection against anaphylaxis was dose dependent in both IL-4Rα^{-/-} and CD4⁺ T-cell specific IL-4Rα^{-/-} mice, and that CD4⁺ T-cell specific IL-4Rα^{-/-} mice were more protected than IL-4Rα^{-/-} mice. As CD4⁺ specific T-cell IL-4Rα^{-/-} mice consistently had high numbers of IFN-γ producing cells at the time of antigenic challenge, it was hypothesized that IFN-γ was able to protect mice against anaphylaxis even in the presence of an established Th2/type 2 response. Indeed, IFN-γ depletion led to a phenotypic switch to lethal anaphylaxis, similar to that seen in wildtype littermates. IFN-γ was neutralized only when sensitization had been completed in order to specifically target the effector phase of the immune

response. The treatment did not significantly alter levels of IL-4 or IL-13 produced by restimulated splenocytes or serum antibody levels. Susceptibility to anaphylaxis after anti-IFN- γ treatment correlated with a significant increase in mucosal mast cell protease levels in the serum and intestinal tissue, normalising levels to those seen in wildtype mice.

Several studies show *in vitro* inhibition of mast cell degranulation by IFN- γ , mediated by nitric oxide (NO)^{70, 71}. The source of this NO is thought to be accessory cells such as macrophages, as it is known that IFN- γ binds to the glycosaminoglycan dermatan sulfate on mast cells, and is then presented to the IFN- γ receptor on macrophages to induce NO⁷². The loss of resistance to anaphylaxis in anti-IFN- γ treated mice was associated with a six-fold increase in serum MMCP-1, a marker of mast cell degranulation. In this model unlike in the model used by Strait et al³³, IgG-dependent anaphylaxis was not associated with suppression of mast cell degranulation and mast cell degranulation occurred in anti-IgE treated mice, indicating that IgG-mediated mast cell degranulation may have occurred. Previous studies have shown that a functional Fc ϵ RI is not required for mast cell degranulation²³ and that Fc γ RIII-dependent mast cell degranulation can occur⁷³. Preliminary data from an experiment where partial mast cell depletion was achieved indicates that mast cells may mediate anaphylaxis in this model. Therefore, it is feasible that IFN- γ reduced the severity of anaphylaxis by inhibiting mast cell degranulation. Recently it has been shown that MMCP-1 directly increases intestinal permeability³². The observed association between fatality of anaphylaxis and intestinal oedema indicates that the suppression of MMCP-1 levels may provide a mechanism through which IFN- γ reduces the severity of anaphylaxis.

In humans, successful allergen-specific immunotherapy is often associated with increased IFN- γ and IL-10, rather than a decrease in Th2 cytokines⁷⁴⁻⁷⁶. Recombinant IFN- γ has been used in clinical trials to treat atopic eczema, where it proved effective at alleviating skin symptoms and led to an unexpected improvement in concurrent rhinitis⁷⁷⁻⁸⁰. The therapy did not decrease IgE levels, but was associated with a decrease in eosinophil and

lymphocyte counts^{77, 78, 80}. Our data suggest that more studies are needed on the effector mechanisms of IFN- γ in allergic responses, as CD4⁺ T cell IL-4R α -/- mice were protected against anaphylaxis even in the presence of IgE, IgG1 and Th2 cytokines.

Several factors could contribute towards the high levels of IFN- γ found in CD4⁺ T-cell specific IL-4R α deficient mice. Anti-CD4 treatment indicated that the primary source of IFN- γ was CD4⁺ cells. Naïve CD4⁺ T-cells in these mice are unable to respond to IL-4 and IL-13 signaling⁴³, and are therefore likely to differentiate into Th1 cells. Non-CD4⁺ T cells bearing the IL-4R α remain IL-4/IL-13 responsive, and IL-4 is known to instruct dendritic cells to produce IL-12, which would strengthen the Th1 pathway^{81, 82}. Raised numbers of IFN- γ producing cells and increased IFN- γ production in CD4⁺ T-cell specific IL-4R α -/- mice were found not only in our model, but also in other disease models studied in our laboratory, including Leishmaniasis and Schistosomiasis^{43, 83}. Despite experiments designed to explain this, the reason is as yet unclear.

Future experiments will attempt to clarify whether anaphylaxis in this model is mediated by mast cells or macrophages. At present, the data show that IFN- γ depletion restores *in vivo* mast cell degranulation and anaphylactic reactions in generally resistant CD4⁺ T-cell specific IL-4R α -/- mice. This strongly suggests a protective role for IFN- γ at the effector level in anaphylaxis and highlights the importance of IL-4R α signalling on CD4⁺ T-cells in anaphylactic pathways.

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CHAPTER FOUR

MECHANISMS OF *ANISAKIS*-INDUCED DERMATITIS AND ASSOCIATED SYSTEMIC SENSITIZATION

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CHAPTER 4: Mechanisms of *Anisakis*-induced dermatitis and associated systemic sensitization

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4.1 SUMMARY

Background: Larvae of the roundworm *Anisakis* are commonly found parasitizing fish, and can cause dermatitis in people frequently handling or exposed to fish, such as seafood processing workers.

Methods: A murine model was established to study local pathology and systemic sensitization following epicutaneous exposure to *Anisakis* larval antigens. Biologically relevant sensitization was assessed by intravenous challenge with *Anisakis* proteins. Several different gene deficient mice were used to characterize the roles of IL-4, IL-13 and the IL-4R α , while recently generated CD4⁺ T-cell specific IL-4R α deficient (Lck^{cre}IL-4R α ^{-lox}) and macrophage/neutrophil specific IL-4R α deficient (LysM^{Cre}IL-4R α ^{-lox}) mice were used to determine cell-specific requirements for IL-4/IL-13 responsiveness.

Results: Epicutaneous sensitization with *Anisakis* larval antigens induced local inflammation, epidermal hyperplasia, Th2 cytokines, and production of anti-*Anisakis* IgE and IgG1. Intravenous challenge of sensitized mice resulted in anaphylactic shock. Skin inflammation was IL-4 independent but IL-13 dependent, while systemic anaphylaxis reactions were partially IL-4 dependent. Macrophage/neutrophil specific expression of the IL-4R α was not required for anaphylaxis but exacerbated skin inflammation. T-cell specific IL-4R α deficient mice experienced partial inhibition of systemic anaphylaxis, despite showing skin inflammation comparable to that of wildtype mice. This illustrates the necessity for cell specific evaluation of IL-4/IL-13 responsiveness during allergic responses.

Conclusions: IL-4, IL-13 and the IL-4R α play important and distinct roles in *Anisakis*-induced dermatitis and associated systemic sensitization in mice. Cell-specific IL-4R α ^{-/-} mice may aid in the elucidation of the mechanisms of eczema and systemic sensitization through the skin.

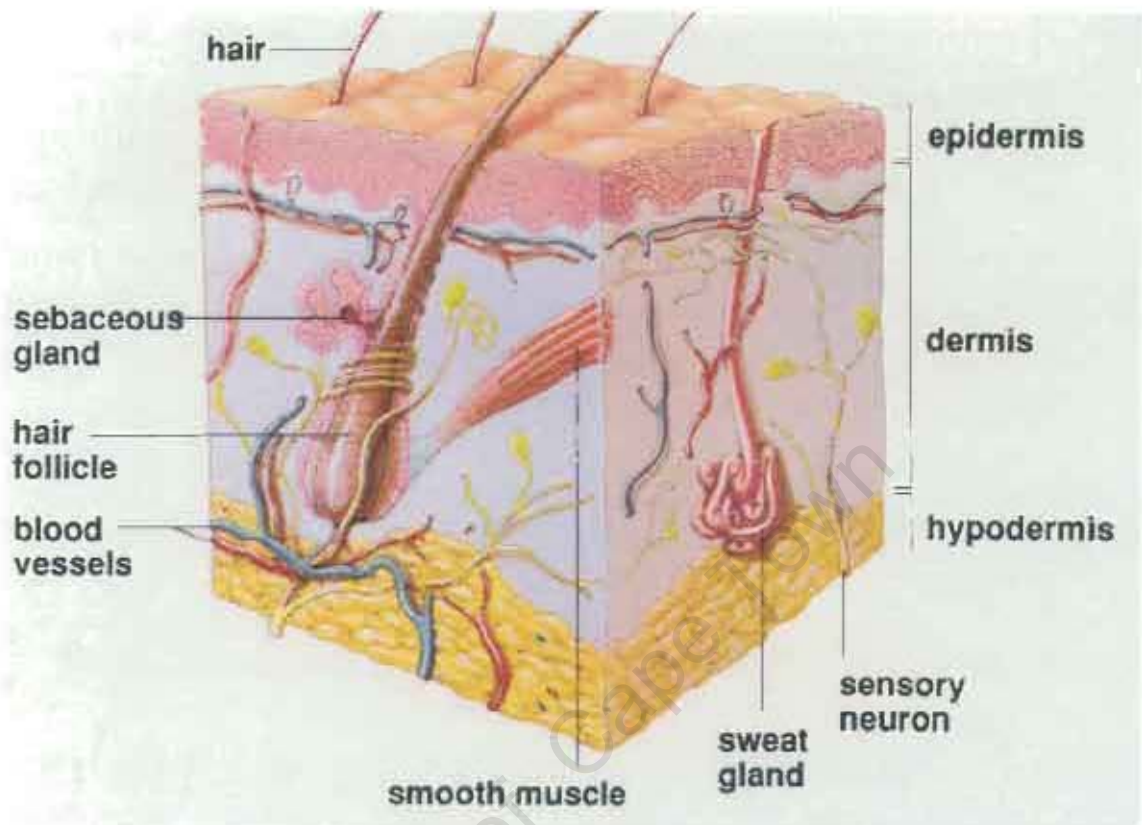


Figure 4.2.1. Structure of the skin. The top layer of the skin is called the epidermis, and provides a barrier against injury and harmful microorganisms. The epidermis consists of several layers of cornifying epidermal cells (keratinocytes). The lowest layer is the stratum germinativum or stratum basale, made up of columnar epithelial cells which gradually grow closer to the surface and cornify (keratinize) to form the stratum corneum, the hardened topmost layer of the skin. Below the epidermis is the dermis, composed of connective tissue and penetrated by sebaceous glands, sweat glands, hair follicles, nerves, blood vessels and lymph ducts. Oil secreted by the sebaceous glands helps to lubricate the skin and contains antimicrobial molecules that help protect against infection. Finally, below the dermis is the hypodermis, containing subcutaneous fat which provides insulation and a cushion for the internal organs. Figure from: www.gen.ufl.edu/~chyn/age2062/lect/lect_19/174.gif.

4.2 INTRODUCTION

Anisakis simplex has been implicated in allergic skin disease, including urticaria, contact dermatitis and atopic dermatitis¹⁻⁷. In a recent cross-sectional epidemiological study of 578 fish processing workers performed by the Occupational and Environmental Health Unit of the University of Cape Town, workers sensitized to *Anisakis* were almost twice as likely to report skin symptoms upon direct contact with fish than non-sensitized workers (Odds Ratio (OR) 1.9; Confidence Interval (CI), 1.0-3.6)⁷. Not all workers sensitized to *Anisakis* demonstrated allergic symptoms, but atopic workers (positive SPT to one or more inhalant allergens) had a 3-fold or more increased risk of presenting with an *Anisakis*-related allergic disease than non-atopic workers.

This indicates that workers frequently handling or exposed to fish could be at an increased risk of becoming sensitized to *Anisakis*, and that sensitization is a risk factor for allergic disease. Possible routes of sensitization include ingestion of infested fish^{1,3}, inhalation of airborne *Anisakis* allergens^{8,9} or direct contact with *Anisakis* proteins in fish^{5,8}. As it has never been directly demonstrated that direct contact with *Anisakis* proteins can cause sensitization, our laboratory decided to attempt to set up an experimental mouse model of *Anisakis*-induced dermatitis.

4.2.1 Allergic skin disease

The skin forms an important physical and protective barrier between the body and the surrounding environment, and encounters a large number of antigens at its surface¹⁰ (Fig 4.2.1). In order to deal with these antigens the skin relies on components of the innate and adaptive immune system, both well-represented in this tissue¹¹ (Fig. 4.2.2). Responses are regulated by a wide array of cytokines and chemokines¹². If dysregulation occurs, allergic disease may result. Allergic diseases of the skin can be broadly divided into urticaria, allergic contact dermatitis and eczema/atopic dermatitis^{11,13}.

4.2.2. Urticaria

Urticaria is an outbreak of cutaneous wheals caused by transient, localized areas of oedema within the skin due to mast cell degranulation and histamine release, and may be

acute or chronic ^{11, 14, 15}. Chronic urticaria refers to urticaria that has recurred at least twice a week for at least 6 weeks. The wheals initially have pale centres surrounded by erythema, subsequently becoming more uniformly pink in colour. A wheal is usually pruritic, and resolves within 24 hours of onset in most cases ^{11, 14}. When oedematous lesions affect the deeper layers of the skin or the mucous membrane tissue, they are referred to as angioedema. This can be dangerous if it affects the oropharynx. Current treatment, in order of preference, includes avoidance of triggers, non-sedating anti-histamines, sedating anti-histamines and immunosuppressives such as corticosteroids and cyclosporine ¹¹.

Factors that can trigger mast cell degranulation within the skin to cause urticaria include physical factors such as cold, heat, sunlight and pressure, medications, infections, foods, and chronic inflammatory, autoimmune or systemic diseases. Approximately half of chronic urticaria cases are idiopathic ^{11, 14, 15}. In about 30-50% of patients, autoantibodies against the alpha subunit of the high affinity IgE receptor (FcεRI) are implicated ^{11, 16, 17}. Interestingly, a recent study found that *Anisakis* was a leading cause of chronic idiopathic urticaria in an endemic area ³. Previous studies had associated *Anisakis* sensitization with acute outbreaks of urticaria due to ingestion of fish and other seafoods ^{1, 2, 4}.

4.2.3 Contact dermatitis

Contact dermatitis refers to an eczematous skin disease characterized by inflammation and itching ¹¹. It primarily affects adults, can be initiated by a wide range of irritants and allergens, and is particularly important in the occupational setting. In many countries it is the leading cause of occupational disease, and is a major cause of lost working time and morbidity, especially when chronic ^{11, 18, 19}. Combined with medical expenses, this places financial strain on both the patient and the employer.

Contact dermatitis can be broadly divided into irritant/nonallergic contact dermatitis and allergic contact dermatitis ^{11, 13, 18}. Irritant contact dermatitis (the more common disease) is caused by antigen non-specific cytotoxicity, while allergic contact dermatitis is the result of an antigen-specific lymphocyte-mediated hypersensitivity reaction, also known

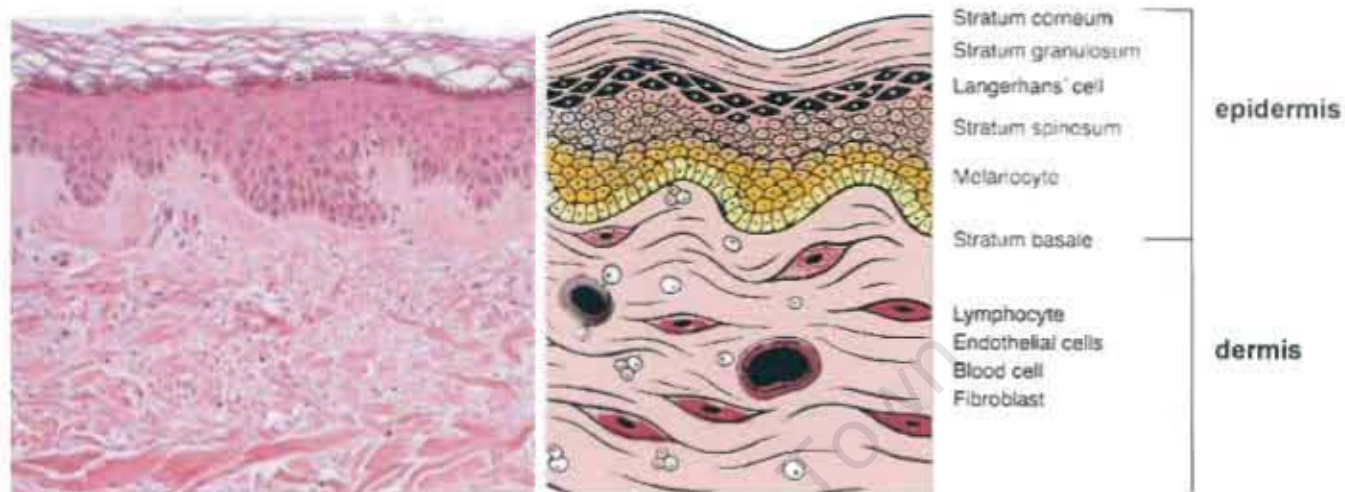


Figure 4.2.2 **Immune cells in the skin.** Antigen-presenting cells called Langerhans cells inspect the epidermis for antigens that may penetrate the protective upper layers of the epidermis through mechanical damage. The dermis, underlying the epidermis, is infiltrated by blood and lymph vessels through which immune cells such as lymphocytes, mast cells and eosinophils may enter the skin. Figure modified from: <http://melanoma.blogspot.com/2006/03/> and www.healthandage.com/html/res/primer/skin.htm.

as contact sensitivity (CS). CS has been considered a subtype of delayed type hypersensitivity (DTH) as both are T-cell mediated^{11,20,21}. However, recently it has been shown that the mechanisms involved in CS and DTH are different^{21, 22}. DTH is dependent on CD4⁺ T cells, while CS is dependent on CD8⁺ T cells with CD4⁺ T cells performing a regulatory function^{22, 23}. Furthermore, antigens that cause DTH are typically large, soluble proteins, while CS is caused by haptens such as small, hydrophobic, electrophilic molecules or metal ions (e.g. nickel) that become immunogenic after binding to host proteins or peptides^{11,24,25}.

Most contact allergens are also irritants¹⁸, and seem to provoke a cascade of pro-inflammatory cytokines that causes activated Langerhans cells or dermal dendritic cells to migrate to the skin draining lymph nodes, where they mediate the differentiation of naïve T cells into memory CD8⁺ T cells^{11, 22, 24}. Memory CD8⁺ T cells circulate in the blood until a second exposure to the sensitizing antigen, which causes them to home to the antigen-exposed skin. The cytotoxicity of the CD8⁺ effector cells appears to play the key role in causing contact dermatitis, as well as their production of large amounts of IFN- γ which stimulates keratinocyte apoptosis^{11, 26}. CD4⁺ T cells are thought to play a regulatory role²³. In mice, CD4⁺ Th2 cells downregulate inflammation in CS in a manner that appears to be regulated by Th2 cytokines such as IL-13²⁷, while CD4⁺ Th1 cells contribute to the pathology of contact dermatitis by increasing inflammation²⁸.

Occasionally the literature refers to “protein contact dermatitis”. This is an IgE-associated disease caused by the absorption of protein through damaged skin, and is therefore likely to be a form of eczema and not a subset of allergic contact dermatitis, which is known to be mediated by CD8⁺ T cells and Th1 cells^{13,29,30}.

4.2.4 Atopic dermatitis/eczema

Atopic dermatitis (AD) has been described as a “chronic, relapsing inflammatory skin disease that is characterized by pruritic eczematous lesions”³¹. It can appear in mild forms (a few dry eczematous patches) to severe forms (extensive erythematous rash) and is often associated with other allergic diseases such as rhinitis and asthma^{11, 32}. Ingestion of

food allergens and exposure to aeroallergens are two common causes of eruptions³²⁻³⁶. Around 70-80% of adult patients have AD that is associated with IgE production against environmental allergens, labelled “extrinsic AD”, while the remaining 20-30% have low IgE levels, do not show sensitization to allergens, and are classified as having “intrinsic AD”³⁷. Recently the term “eczema” has been proposed by the World Allergy Organization to replace “atopic dermatitis”, with the term “extrinsic AD” to be replaced by “atopic eczema” and the term “intrinsic AD” to be replaced by “nonatopic eczema”¹³. This is perhaps a better terminology, as the term “atopic dermatitis” may be slightly misleading in that not all sufferers are atopic. Therefore from hereon the new terminology will be used.

Eczema often begins in infancy, frequently in individuals with a family history of atopy, and resolves around puberty but may reoccur in adulthood³¹. While eczema can also appear for the first time in adulthood, individuals with eczema in early life are at an increased risk of developing work-related eczema later on^{11, 18}. The lifetime prevalence of eczema is 1-3% in adults and 10-20% in children³⁸.

Eczema is a genetically complex, familiarly transmitted disease³². Genetic linkage studies have identified several chromosomal regions with linkage to eczema³⁹. These include regions which contain a clustered family of Th2 cytokine genes (IL-3, IL-4, IL-5 and IL-13), and genes believed to be involved in skin inflammation, amongst others. Eczema patients frequently have intrinsic defects in the lipid composition of the skin that result in increased transepidermal water loss, severe dryness, and impairment of the barrier function of the skin (reviewed by Novak et al. 2003)³². In the working environment, “wet work” or chronic friction can cause similar disruption of the epidermal barrier. In both cases, the result is a higher permeability to irritants and allergens as well as increased susceptibility to microbes such as *Staphylococcus aureus* and yeasts that contribute to inflammation in the skin^{11, 40, 41}. The mechanical damage to the skin caused by scratching during the active inflammatory phase of eczema (which is accompanied by severe pruritis) further disrupts the skin barrier and triggers release of various inflammatory mediators, making eczema a self-perpetuating disease³². Furthermore, in

severe eczema certain intracellular proteins released from cells through mechanical damage can function as autoallergens, exacerbating the inflammation⁴².

4.2.4.1 The burden of eczema

Spergel and Paller (2003) review the effect of eczema on the quality of life in infants, children and adolescents⁴³. Chronic itching and scratching as well as disruption of sleep combine to adversely affect school performance, sports and social relationships. Children with eczema frequently display psychological abnormalities, behavioural problems, mood changes and hyperactivity, anxiety, distress, poor-self esteem, lack of self-confidence and embarrassment over their disfiguring lesions. The disease also places a burden on the caregivers and families of patients, resulting in stress and disruption of sleep in the caregiver, family dysfunction, and financial strain due to medical expenses, the cost of special food, bedding or clothes, and interference with the employment of the caregiver.

Occupational skin disease can also strongly impact the quality of life and is the most frequently reported occupational disease in many countries⁴⁴. Workers may require a change of environment if the disease is due to an allergen that cannot be avoided or if the job involves repeated, unavoidable exposure to cutaneous irritants such as water, soaps and solvents⁴⁵. If alternative jobs are not available this can result in unemployment. Food-related occupational eczema has been associated with a higher risk of job loss, particularly as hygiene regulations may restrict people with hand eczema from working with food⁴⁶. Sensitization to common environmental allergens such as nickel, rubber or foods means that the disease can persist even after removal from the working environment¹⁸. Furthermore, a delay in treatment can allow the dermatitis to develop into an endogenous, self-perpetuating disease from which recovery is limited, even if the original sensitizing agents are avoided^{18,42}.

4.2.4.2 The atopic march

Eczema is often the first manifestation in the typical age-related sequence of atopic diseases known as the “atopic march”⁴³. It commonly occurs in the early years of life and then regresses, while respiratory diseases manifest later. Eczema occurs within the

first 5 years in 85% of individuals, and within the first year in 60% of individuals⁴⁷. Approximately half of all eczema patients go on to develop asthma, while as many as two-thirds develop allergic rhinitis⁴³. The severity of eczema has been related to the likelihood of developing later respiratory allergy^{48,49}.

As the clinical signs of eczema pre-date the development of other allergic diseases, it has been suggested that eczema could be an “entry point” for subsequent allergic disease, with cutaneous exposure acting as an important route of sensitization to allergens^{27,43,50,51}. The fact that one of the cardinal features of eczema is a genetic defect in skin barrier function which allows for penetration of allergens into the immune network lends support to this theory³⁸. Lack et al found that atopic children exposed to peanut oil in baby creams had an increased risk of developing peanut allergy⁵¹. In another study, non-asthmatic patients with eczema and sensitization to house dust-mites (HDM), demonstrated airway hyperreactivity to HDM challenge, suggesting that the skin could be a site at which primary sensitization occurs⁵². Similarly, in mouse models, epicutaneous application of ovalbumin (OVA) results in elevated serum IgE levels, Th2-skewed responses and airway hyperresponsiveness to OVA^{27,53}, while epicutaneous sensitization with *Aspergillus fumigatus* strongly predisposes mice to nasal responses⁵⁴.

It is thought that memory Th2 cells generated through epicutaneous exposure to allergens circulate in the body, passing through sites such as the lungs and nasal mucosa^{27,43}. Eczema patients have increased numbers of Th2 cells and decreased numbers of Th1 cells in the peripheral blood⁵⁵. Inhalation of allergens could activate these memory cells, resulting in an environment rich in Th2 cytokines²⁷. Presentation of allergen by dendritic cells to naïve T cells in such an environment would cause further Th2 skewing^{27,56}. Together this would promote allergic responses such as eosinophilia, mucus hypersecretion and mast cell proliferation. If the cutaneous route of exposure to allergens does play a primary role in the development of allergic diseases, it is possible that intervention at the eczema stage could prevent or reduce subsequent forms of allergic disease such as asthma, a disease which can be life-threatening and for which there is no cure⁴³. Already, recent trials have investigated the effects of anti-histamine

administration on preventing the development of asthma in eczema patients, with promising results in certain subgroups of patients (reviewed by Spergel 2003)⁴³. In order to optimize preventative therapies, it will be necessary to understand the mechanisms controlling the sensitization process²⁷.

4.2.4.3 The immunopathology of eczema

Immunologically, eczema is characterized by inflammatory cell infiltration and upregulation of inflammatory cytokines in the skin, accompanied by raised levels of serum IgE in the case of atopic eczema⁵⁷. In acute lesions the infiltrate is composed primarily of CD4⁺ memory T cells, while in chronic lesions there is a marked inflammation composed of eosinophils, lymphocytes, monocytes/macrophages, Langerhans cells, inflammatory dendritic epidermal cells (IDECs) and mast cells, accompanied by epidermal hyperplasia, spongiosis, dermal thickening and parakeratosis^{11, 31, 32, 57, 58}. Both atopic and nonatopic eczema have associated eosinophilia³⁸.

The process of eczema is thought to begin when allergens entering a barrier-disrupted skin are carried to the skin-draining lymph nodes by antigen presenting cells such as Langerhans cells or IDECs³². Here the antigen presenting cells cause the differentiation of naïve T cells into CD4⁺ memory T cells with the skin homing receptor CLA³⁸. These cells circulate until a second encounter between the skin and the sensitizing antigen. In acute atopic eczema, Th2 skewing is seen, with low levels of IFN- γ and high levels of IL-4, IL-5 and IL-13 produced in the skin, and increased numbers of IL-4 and IL-5 producing Th2 cells in the blood^{11, 38}. In contrast, less IL-4 and IL-13 is produced in nonatopic eczema³⁸.

Upon a second exposure to antigen, Th2 cells release an array of cytokines and chemokines that cause infiltration of cells into the skin, including lymphocytes,

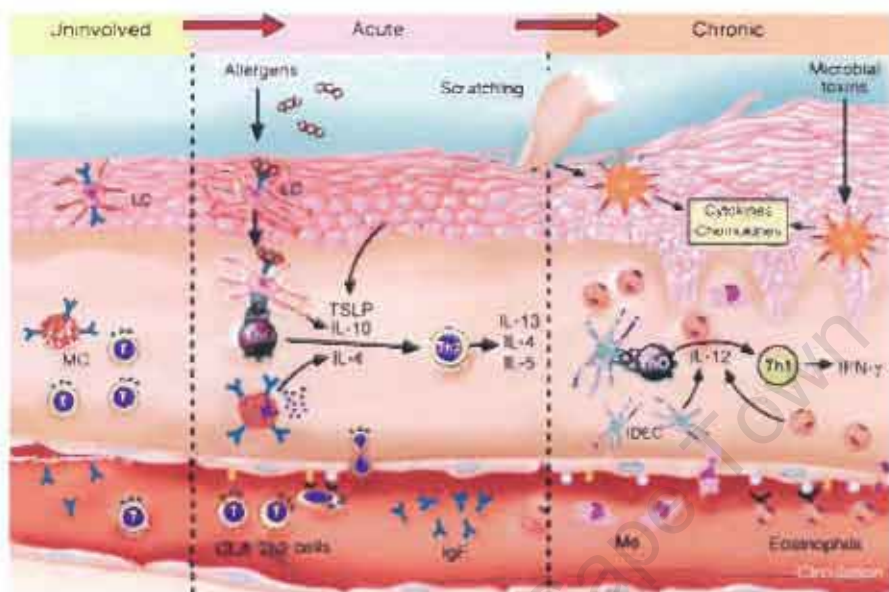


Figure 4.2.3 Immune mechanisms in eczema. From Leung (2004)³⁸. T cells expressing the skin homing receptor CLA circulate through the skin. In the presence of IL-4, Th2 differentiation occurs, resulting in the recruitment of effector cells and the release of inflammatory mediators from mast cells. Scratching and exposure to microbial toxins activates keratinocytes to release proinflammatory cytokines and chemokines that facilitate the extravasation of inflammatory cells into the skin, including IDECs, macrophages (Mφ) and eosinophils. Inflammatory cells produce IL-12, resulting in a switch to a Th1-cytokine environment with increased IFN- γ expression.

eosinophils, mononuclear cells, dendritic cells and mast cells. Chronic eczema is associated with increased numbers of inflammatory cells that produce IL-12, resulting in a switch to a Th1 cytokine environment with increased IFN- γ production³⁸ (Fig. 4.2.2). Chronic lesions undergo tissue remodelling with collagen deposition, resulting in the formation of thickened, lichenified plaques and dry fibrotic papules³⁸. These lesions have fewer IL-4 and IL-13 mRNA expressing cells, but more IL-12, IFN- γ , IL-5 and GM-CSF expressing cells than in acute AD^{11, 38}. Overall, studies in mice have indicated that both Th1 and Th2 cytokines play important roles in dermatitis, as do a wide variety of chemokines (e.g. eotaxin, RANTES, TARC) that stimulate migration of inflammatory cells into the skin^{11, 32, 53, 59}.

4.2.4.4 IL-4 and IL-13 in eczema

Specific polymorphisms in the genes for IL-4, IL-4R α and the Th2-associated transcription factor STAT6 are significantly associated with eczema^{39, 57, 60, 61}. However, STAT6^{-/-} mice retain the ability to develop eczema despite defects in Th2 responses^{54, 62}. Recently, an association has been found between distinct polymorphisms in the IL-4R α and atopic versus nonatopic eczema⁶¹. Together these data suggest that IL-4, IL-13 and/or the IL-4R α may be of special importance in the atopic form of eczema, which is associated with other atopic diseases such as allergic rhinitis, asthma and food allergies³⁷. This would indicate a particular importance in sensitization to allergens and associated allergic responses, in addition to any possible role in the pathology of AD lesions.

Mechanisms of eczema are currently being investigated in various laboratories using OVA-based models. These models commonly involve repeated epicutaneous application of OVA, resulting in local dermatitis with infiltration of eosinophils and CD4⁺ T cells, increased Th2 mRNA expression in the skin and raised levels of total and specific IgE in the serum⁵³. In these ways this model successfully mimics atopic eczema, and is therefore useful for elucidating the roles of various cytokines and chemokines in this disease. To date, several publications have explored the roles of IL-4 and/or IL-13 in murine models of eczema.

Spergel et al found that eotaxin mRNA expression and eosinophil infiltration was decreased in the sensitized skin of IL-4 $-/-$ mice, while numbers of infiltrating T cells, particularly CD4⁺ T cells, were greater⁶³. Serum IgE levels were drastically reduced, while IgG2a levels were increased, and Th1 cytokine expression was increased in the skin. However, IL-4 $-/-$ mice had normal thickening of the skin and equivalent blood eosinophil levels when compared to wildtype mice. Interestingly, transgenic mice constitutively expressing IL-4 in the basal epidermis spontaneously developed a skin disease resembling chronic eczema in humans, complete with IgE production⁶⁴. One could therefore speculate that dermatitis in IL-4 $-/-$ mice resembles nonatopic eczema in humans, where inflammatory skin disease is present without raised levels of serum IgE, whereas eczema in the presence of IL-4 more resembles atopic eczema.

Herrick et al investigated the role of IL-4 and IL-13 in the generation of Th2 responses and airway hyperreactivity following epicutaneous sensitization^{27, 65}. They found that IL-4 $-/-$ mice maintained airway inflammation (increased total cells and eosinophils in BAL, mucus hypersecretion) and Th2 activation (cells producing IL-5 and IL-13 in the lungs) after inhalational challenge, though IgG2a was increased and IgE and IgG1 decreased as in the research by Spergel et al.⁶³. This was in contrast to mice that were initially sensitized by the inhalational route, which showed dependence on IL-4 for Th2 responses and airway hyperreactivity. Additional studies showed that lung inflammatory responses, IgG1 production and IL-5 produced by skin-draining lymph nodes were decreased in IL-13 depletion and IL-13 $-/-$ mice. This led the authors to conclude that IL-13 was the key cytokine involved in generation of Th2 activation after epicutaneous sensitization^{27, 65}.

4.2.5 Aims of the current study

The first aim of the current study was to determine whether *Anisakis* proteins are able to cause dermatitis and/or systemic sensitization through epicutaneous exposure. To date, models of eczema or protein contact dermatitis have primarily used OVA, and it has been shown that responses to other allergens or to crude extracts can differ from responses to OVA⁵⁴. Until recently it was assumed that inhalational exposure to allergens was the cause of sensitization leading to diseases such as rhinitis and asthma⁶⁵. However since

recent data has indicated that epicutaneous exposure could lead to respiratory hyperreactivity, it is important to determine how relevant epicutaneous sensitization is in terms of the risk of developing allergic disease. In terms of *Anisakis* exposure, fish processing workers are at particular risk, as are people who frequently prepare fish in the domestic environment. In the course of a study of occupational diseases in local seafood processing factories undertaken by the Occupational and Environmental Health Unit of UCT, it became clear that not all workers use gloves while processing fish. Reasons for this include discomfort caused by sweating inside the gloves or by liquids entering the gloves while working, allergy to the gloves themselves, or reduced ease in handling fish or processing machines. In addition, workers who wear gloves still often have bare arms and can easily be splashed with fish juices containing various allergenic proteins. In the domestic environment, people do not usually wear gloves when handling fish. Many people I have spoken to have reported seeing worms while preparing snoek, a fish that is eaten locally and is commonly infested with *Anisakis*.

The second aim of the study was to further elucidate the differential roles of IL-4 and IL-13 in local pathology and systemic sensitization caused by epicutaneous allergen exposure, and in addition to determine the role of the IL-4R α in these processes. As the immunopathogenesis behind protein-induced dermatitis remains unclear, the importance of IL-4R α expression on specific cell types was also investigated. Previous models of eczema in mice have demonstrated that cells infiltrating into the dermis are predominantly CD4⁺ T cells and macrophages⁵³. Therefore the role of IL-4R α expression on CD4⁺ T cells and on macrophages was investigated using CD4⁺ T cell specific IL-4R α ^{-/-} mice (Lck^{Cre}IL-4R α ^{-/lox}) and macrophage/neutrophil specific IL-4R α ^{-/-} mice (LysM^{Cre}IL-4R α ^{-/lox}).

4.3 METHODS

Mice

BALB/c mice (8-10-wk-old) and IL-4^{-/-} ⁶⁶, IL-13^{-/-} ⁶⁷, IL-4/13^{-/-} ⁶⁸, IL-4R α ^{-/-} ⁶⁹, CD4⁺ T cell specific IL-4R α ^{-/-} (Lck^{Cre}IL-4R α ^{-lox}) ⁷⁰ and macrophage/neutrophil specific IL-4R α ^{-/-} (LysM^{Cre}IL-4R α ^{-lox}) ⁷¹ mice (all BALB/c background) were used from in-house breeding colonies. All mice were housed under SPF barrier conditions using individual ventilated cages (IVC) in the animal facility at the University of Cape Town, South Africa. All experiments complied with the South African Code of Practice, and were approved by the University of Cape Town Ethic Research Committee (ERC). Mice were genotyped by PCR by staff of the Division of Immunology.

Anisakis extract

Anisakis pegreffii larvae (L3) were collected from the intestines of parasitized fish (*Thyrsites atun*) with forceps, and extensively washed in 4% acetic acid and phosphate-buffered saline (PBS) (Appendix A). The larvae were frozen, homogenized in PBS and sonicated to lyse cells. The extract was pipetted into 2ml eppendorfs and centrifuged at 14000rpm for 20-30 minutes to remove particulate matter, then filter-sterilized through a 0.20 μ m filter (Sartorius, Goettingen, Germany). Protein concentration was measured by BCA test (Pierce, Rockford, Illinois, U.S.A.) according to the manufacturer's protocol.

Sensitization schedule

Mice were anaesthetized with 200 μ l of ketamine (Anaket-V; Centaur Labs, Johannesburg, South Africa)/ xylazine (Rompun; Bayer, Isando, South Africa) anaesthetic (Appendix A) and a small area of the abdomen was shaved. A volume of 200 μ l containing 200 μ g of filter-sterilized *Anisakis* protein extract was applied to a 1 x 2cm gauze patch secured to the skin with transparent hypoallergenic plaster (Band-Aid[®], Johnson and Johnson, Australia). After one week the patches were removed. Two weeks later a second patch was applied in the same location. A total of three patches were applied, separated by two-week intervals.

A shortened version of the protocol was found to yield the same results, and this protocol was subsequently used in experiments in which the endpoint was an intravenous challenge. In this protocol, the first patch was applied for 5 days, followed by a 5 day rest period. This was repeated with a second patch. Finally, a third patch was applied for 5 days, following which mice were challenged intravenously with 750µg of *Anisakis* extract.

Temperature measurements

Rectal temperature readings were taken every 10 minutes using a mouse rectal thermometer (VetTech, Cheshire, U.K.) lubricated with VaselineTM petroleum jelly (Unilever, La Lucia, South Africa).

Anaphylaxis scoring system

Mice were observed for 60-70 minutes post challenge, and signs of anaphylaxis were scored using a modification of a previously described scoring system⁷². 0, no symptoms; 1, irritability, scratching and/or hypersensitivity to touch; 2, reduced activity, pilar erecti, and/or diarrhoea; 3, lying flat but upright, weakness and/or cyanosis around the mouth and tail; 4, lying on side, no activity upon prodding, loss of grip, loss of consciousness with or without convulsions; 5, death. Mice beginning convulsions invariably died soon after, and were therefore euthanased for humane reasons and scored as 5.

Histology

Skin taken from the site of epicutaneous exposure was preserved in 4% phosphate-buffered formalin for at least 24 hours and then embedded in wax. Paraffin sections of 5µm were cut and fixed to slides, and stained with haemotoxylin and eosin.

Anti-IgE staining

Tissue samples were embedded in Tissue-Tek oxacalctriol (OCT) compound (Sakura Finetek U.S.A. Inc., Torrance, California, U.S.A.), frozen on dry ice and stored at -80°C. Sections of 5µm were cut using a Leica LM 1850 Cryostat (Leica Instruments, Nusstoch, Germany), placed onto slides and dried overnight at 4°C. The slides were fixed in acetone

for 10 minutes at room temperature and allowed to air-dry for 5 minutes, then washed 4x5 minutes in PBS (Appendix A). Following this the sections were blocked for 30 minutes at room temperature in 10% rat serum diluted in PBS. The slides were rinsed in PBS, then 100µl of 10µg/ml PE-labelled anti-IgE (clone 2.3G3, Southern Biotechnology, Birmingham, Alabama, U.S.A.) diluted in PBS was placed onto the sections and the slides were incubated for 90 minutes at room temperature. The slides were washed 4x5 minutes in PBS, mounted using fluorescent mounting medium (Dako Cytomation, Carpinteria, California, U.S.A.) and viewed under a fluorescent microscope.

RNA extraction

Each section of skin was homogenized in 1ml Trizol (Sigma, Steinheim, Germany). The homogenate was mixed with 100µl of 49:1 chloroform/isoamyl alcohol, incubated for 15 minutes on ice and centrifuged for 20 minutes at 12000rpm. The upper aqueous phase was transferred to a new tube and mixed with an equal volume of isopropanol to precipitate the RNA. Samples were placed at -80°C for a minimum of 30 minutes, then centrifuged for 10-30 minutes at 14000rpm. The supernatants were discarded, and the pellets were resuspended in 75% alcohol and incubated at room temperature for 15 minutes. The samples were then centrifuged for 5 minutes at 14000rpm and the supernatants were discarded. The pellets were air-dried for 15 minutes, and then dissolved in 100µl DEPC-treated water (Appendix A). RNA concentration and purity were assessed by taking readings at 200-300nm on a spectrophotometer, following which the RNA integrity was checked by gel electrophoresis.

RNA gel electrophoresis

A formaldehyde denaturing 1% agarose gel was prepared (Appendix A) and placed into a gel tank containing MOPS electrophoresis buffer (Appendix A). Samples were prepared by mixing 2.6µl of RNA (1-2µg) with 7.4µl of loading buffer (see Appendix A) and incubating for 5 minutes at 65°C to heat denature the RNA. The samples were cooled on ice for 5 minutes and loaded onto the gel in parallel with a bromophenol blue dye running marker (Appendix A), then electrophoresed at 40V until the bromophenol blue dye had

migrated approximately 8cm. The RNA was visualized by placing the gel onto a UV transilluminator.

DNase treatment of RNA

DEPC-treated water (Appendix A) was added to 1-10 μ g of RNA to make up a final volume of 48 μ l. The RNA was then mixed with 52 μ l of a DNase I cocktail to provide a final concentration of 1x First Strand Buffer (Gibco, Paisley, Scotland), 1.0U/ μ l Roche RNase-free DNase (Roche, Mannheim, Germany) and 0.4U/ μ l Promega RNAsin (N2511)(Promega Corporation, Madison, Wisconsin, U.S.A.). The mix was incubated at 37°C for 90 minutes, following which the DNase I was heat deactivated by incubation at 65°C for 15 minutes. Purification of RNA was performed using the RNeasy MinElute Cleanup Kit (Qiagen, Hamburg, Germany). The concentration and purity of RNA were assessed by taking readings at 200-300nm on a spectrophotometer, and the RNA integrity was checked by gel electrophoresis. To test that the RNA was free of genomic DNA, PCR was performed using beta-actin primers that bind to and amplify up genomic DNA (5'-TGGAATCCTGTGG CAT CCAGAAAC-3' and 5'-TAAAACGCAGCTCAGT AACAG-3'). Genomic DNA was included in the PCR run as a positive control.

cDNA synthesis

The Promega ImProm-IITM Reverse Transcription System (Promega Corporation, Madison, Wisconsin, U.S.A.) was used to synthesis cDNA. Briefly, 1-5 μ g of RNA was combined with Oligo(dT)15 primer (0.5 μ g/reaction) and DEPC water to a total volume of 20 μ l, and incubated in a 70°C heat block for 5 minutes. The tubes were then chilled for at least 5 minutes and spun in a microfuge to collect the condensate. A 20 μ l volume of reverse transcriptase (RT) cocktail was added to each tube and pipetted up and down to mix. The samples were incubated in a 25°C heat block for 5 minutes to anneal the primers to the mRNA, and then incubated in a 50°C water bath to allow extension to take place. Following extension, the reverse transcriptase was heat deactivated by incubation at 75°C for 15 minutes. The resulting cDNA was aliquoted and stored at -20°C. Working aliquots were stored at 4°C.

PCR

Capillary tubes, caps, tube holders and eppendorfs were UV cross-linked prior to use. A 2 μ l volume of cDNA sample, diluted standard or autoclaved ddH₂O (negative control) was added to 18 μ l of master mix in each capillary tube to yield a final concentration of 1x Sensimix d(I) (Biolabo Scientific Instruments SA, Châtel-St-Denis, Switzerland), 1x SYBR green (Biolabo Scientific Instruments SA, Châtel-St-Denis, Switzerland), 0.5 μ M forward primer (University of Cape Town), 0.5 μ M reverse primer (University of Cape Town), 0.1mg/ml BSA (Promega Corporation, Madison, Wisconsin, U.S.A.) and 1.5mM MgCl₂ (Promega Corporation, Madison, Wisconsin, U.S.A.). The capillary tubes (Roche, Mannheim, Germany) were capped and centrifuged for 10 seconds at 1000rpm to settle the samples. Capillary tubes were put into the Lightcycler (Roche, Mannheim, Germany) and processed according to the following program: 95°C for 10 minutes to activate the enzyme, then 50 cycles of 94°C for 5 sec, 60°C for 10 sec and 72°C for 15 sec, using the following primers: IL-4, 5'-TCGGCATTTCGAAACGAGGTC-3' and 5'-GAAAAGCCCGAAAGAGTCTC-3'; IL-13, 5'-CTCACTGGCTCTGGGCTTCA-3' and 5'-CTCATTAGAAGGGGCCGT GG-3'; IFN- γ , 5'-GCTCTGAGACAATGAACGCT-3' and 5'-AAAGAGATAATCT GGCTCTGC-3'; IL-10, 5'-AGCCGGGAAGACAATAACTG-3' and 5'-CATTTCG ATAAGGCTTGG-3'. Data were analyzed using the "fit points" and "standard curve method" using the β -2-actin as a housekeeping gene.

Collection of serum

Blood samples taken before challenge were collected into serum separator tubes (BD MicrotainerTM SST, BD, Franklin Lakes, U.S.A.) by tail vein bleeding under an infra-red heating lamp. Alternatively, blood was taken from killed mice after cervical dislocation. Blood samples were centrifuged for 15 minutes at 3000 rpm before being placed at -80°C for storage.

MMCP-1 ELISA

MMCP-1 levels in serum were measured by ELISA according to the manufacturer's protocol (Moredun Scientific Ltd, Midlothian, U.K.).

Antigen-specific antibody ELISAs

Nunc Maxisorp ELISA plates (Nunc, Reskilde, Denmark) were coated overnight at 4°C with *Anisakis* extract or OVA in PBS at 5 µg/ml (for IgG1, IgG2a, IgG2b) or 1mg/ml (for IgE), then blocked in ELISA block buffer (Appendix A) for 1 hour at 37°C. Plates were washed 3x with ELISA wash buffer (Appendix A), and serum samples serially diluted in ELISA dilution buffer (Appendix A) were added. Plates were incubated overnight at 4°C, then washed 4x. Alkaline-phosphatase (AP)-labelled goat-anti-mouse IgG1, IgG2a, IgG2b or IgE, 0.5 µg/ml, (Southern Biotechnology, Birmingham, Alabama, U.S.A.) was added for 2 hours at 37°C. Plates were washed 4x and 4-nitrophenyl phosphate disodium salt hexahydrate (PNP) (Sigma, Steinheim, Germany) substrate (see Appendix A) was added. Absorbance was measured at 405nm with 492nm as a reference wavelength, using a VERSAmix tuneable microplate reader (Molecular Devices, Sunnyvale, California, U.S.A.)

Total IgE ELISAs

Sandwich ELISA was performed using purified anti-IgE from clone 84.1C as a coat (1 µg/ml) and AP-labelled goat anti-mouse IgE (Southern Biotechnology, Birmingham, Alabama, U.S.A.) as a secondary antibody, according to the protocol outlined above ("Antigen-specific antibody ELISAs"). Purified recombinant mouse IgE (BD Pharmingen, San Diego, U.S.A.) was used as a standard.

Isolation of splenocytes

Spleens were aseptically removed from mice with forceps, and splenocytes were isolated by pressing through a metal sieve in 10ml Iscove's Modified Dulbecco's Medium (IMDM)(Gibco, Paisley, Scotland). The cells were centrifuged at 1200rpm for 5 minutes, and the pellet was resuspended in 3-5ml of ice-cold red cell lysis buffer (Appendix A). After 2-5 minutes on ice, the cells were centrifuged at 1200rpm for 5 minutes. Pelleted

cells were resuspended in 10ml of IMDM supplemented with 10% FCS (Delta, Kempton Park, South Africa), 2mM L-glutamine (Gibco, Paisley, Scotland), 100µg/ml penicillin (Gibco, Paisley, Scotland) and 100µg/ml streptomycin(Gibco, Paisley, Scotland). The splenocytes were then filtered through a 70µm cell strainer (BD Falcon, Bedford, Massachusetts, U.S.A.) to remove debris, and diluted 1 in 10 to 1 in 30 with Trypan Blue (Sigma, Steinheim, Germany) and PBS for counting.

Splenocyte restimulation

Splenocytes were diluted to 4×10^6 cells/ml with IMDM (Appendix A), and cultured in 48-well plates (Costar, Corning, New York, U.S.A.), with 250µl of cells added to one of the following: 250µl medium, wells pre-coated with anti-CD3 (clone 145-2C11; 10µg/ml) overnight at 4°C to which 250µl of medium had been added; 25µg of conA in 250µl medium, 250µl of 200µg/ml *Anisakis* extract in medium, 250µl of 200µg/ml Grade V ovalbumin (Sigma, Steinheim, Germany) in medium. The cells were incubated at 37°C with 5% CO₂, and supernatants were collected after 48 hours and stored at -80°C.

Cytokine ELISAs

Sandwich ELISAs were performed to determine cytokine levels in cell supernatants. Nunc Maxisorp ELISA plates (Nunc, Reskilde, Denmark) were coated with purified anti-IL-4 (clone 11B11, 2µg/ml), anti-IFN-γ (clone An18KL6, 1µg/ml) or anti-IL-5 (1µg/ml), anti-IL-9 (2µg/ml), anti-IL-13 (1µg/ml), anti-IL-10 (1µg/ml) or anti-TGF-β (0.5µg/ml)(all BD Pharmingen) diluted in PBS, and incubated overnight at 4°C. Plates were blocked with ELISA block buffer (Appendix A) for 1 hour at 37°C, then washed 3x with ELISA wash buffer (Appendix A). Serially diluted standards (purified recombinant IL-4, IL-5, IL-9, IL-13, IFN-γ, IL-10 or TGF-β (all BD Pharmingen, San Diego, U.S.A.)) or cell supernatant, all diluted in ELISA dilution buffer, were added to the plates, and incubated overnight at 4°C. Samples for the detection of TGF-β were pre-treated with acid to unfold the protein for antibody binding (Appendix B). The plates were washed 4x, and biotinylated goat-anti-mouse IL-4 (0.5µg/ml), IL-5 (0.5µg/ml), IL-9 (1µg/ml), IL-13 (0.5µg/ml), IFN-γ (0.5µg/ml), IL-10 (0.5µg/ml) or TGF-β (0.5µg/ml) antibodies (all BD Pharmingen, San Diego, U.S.A.) diluted in ELISA dilution buffer were added for 3 hours

at 37°C. Plates were washed 4x and 1µg/ml AP-labelled streptavidin (BD Pharmingen, San Diego, U.S.A.) was added for 1 hour at 37°C. Finally, plates were washed 4x and PNP (Sigma, Steinheim, Germany) substrate (Appendix A) was added. Absorbance was measured at 405nm with 492nm as a reference wavelength using a VERSAmax tuneable microplate reader (Molecular Devices, Sunnyvale, California, U.S.A.). Cytokine levels in supernatants were determined by reading from the standard curve.

Statistical analysis

Values are given as means +/- SEMs, and significant differences were determined with the unpaired 2-tailed Student *t* test using computer software (GraphPadPrism 4.0, GraphPad Software Inc., San Diego, California, U.S.A.) For allergic score graphs, the Mann-Whitney test was performed for two experimental groups, and the Kruskal-Wallis test with Dunn's post test for three or more groups. Values *P* < 0.05 were considered significant.

University of Cape Town

4.4 RESULTS

4.4.1 Epicutaneous exposure to *Anisakis* extract induces localized dermatitis

Gauze patches containing PBS, OVA or *Anisakis* extract were affixed to small shaved areas on the abdomens of wildtype mice. After epicutaneous application of both *Anisakis* extract and OVA, a localized dermatitis was seen, characterized by epidermal hyperplasia, dermal thickening and infiltration of immune cells (Fig. 4.4.1). The inflammatory infiltrate consisted primarily of mononuclear cells, neutrophils, lymphocytes and eosinophils. No differences were observed between local pathology due to OVA application and *Anisakis* application. Mice which were exposed to PBS alone occasionally showed a very slight increase in epidermal thickness, possibly due to shaving and application of the patch. Staining with anti-IgE revealed the presence of an increased number of IgE-bearing cells in the dermis of *Anisakis*-sensitized mice as compared to PBS controls (Fig. 4.4.2). In addition, *Anisakis*-sensitized mice demonstrated an increase in IL-4 and IL-13 mRNA expression, but no change in IFN- γ or IL-10 mRNA expression, as compared to PBS controls (Fig. 4.4.3).

4.4.2 Epicutaneous exposure to *Anisakis* extract causes systemic sensitization

Following epicutaneous application of *Anisakis* extract, mice had increased levels of *Anisakis*-specific IgG1 and IgE, as well as raised total IgE (Fig. 4.4.4). There was also a slight but significant increase in IgG2b levels. Levels of *Anisakis*-specific IgG2a were barely detectable. Mice epicutaneously sensitized with OVA produced the same pattern of IgG antibodies, although total IgE levels were lower than in *Anisakis*-sensitized mice (Fig. 4.4.5). The higher levels of total IgE in the *Anisakis* model may be due to the ability of helminth proteins to stimulate a polyclonal IgE response⁷³.

4.4.3 Epicutaneous exposure to *Anisakis* extract causes systemic Th2 skewing

Cytokine production was evaluated in skin-draining lymph nodes and splenocytes restimulated with anti-CD3 or antigen (*Anisakis* or OVA) (Fig. 4.4.6). Anti-CD3 restimulated lymph node cells and splenocytes from *Anisakis*- and OVA-exposed mice showed a trend towards increased production of Th2 cytokines, but no difference in production of the Th1 cytokine IFN- γ , compared to PBS- exposed mice. Restimulation of

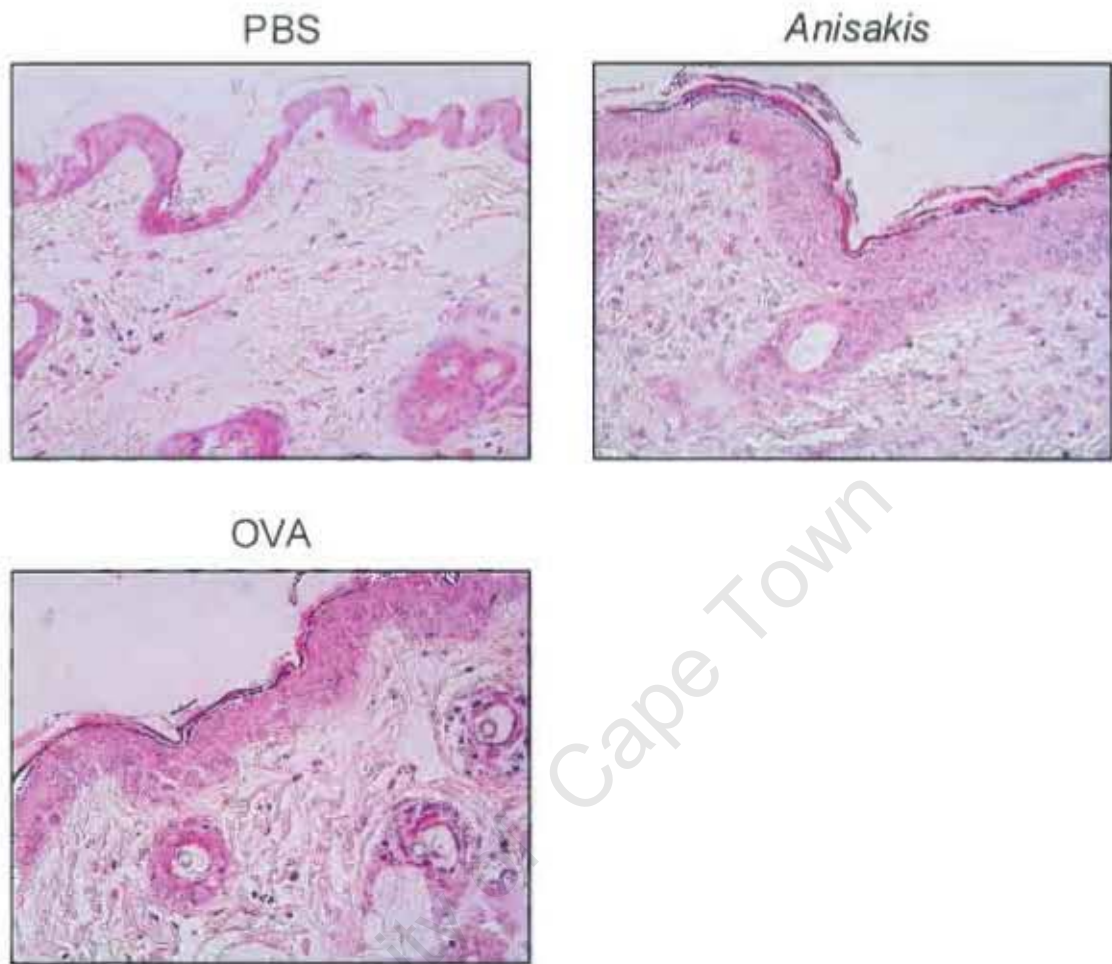


Figure 4.4.1. Histological features of mouse skin epicutaneously exposed to PBS, *Anisakis* extract or OVA. Sections were stained with H&E. Photographs were taken at 400x. The photographs are representative of 2-3 experiments, n = 4-5.

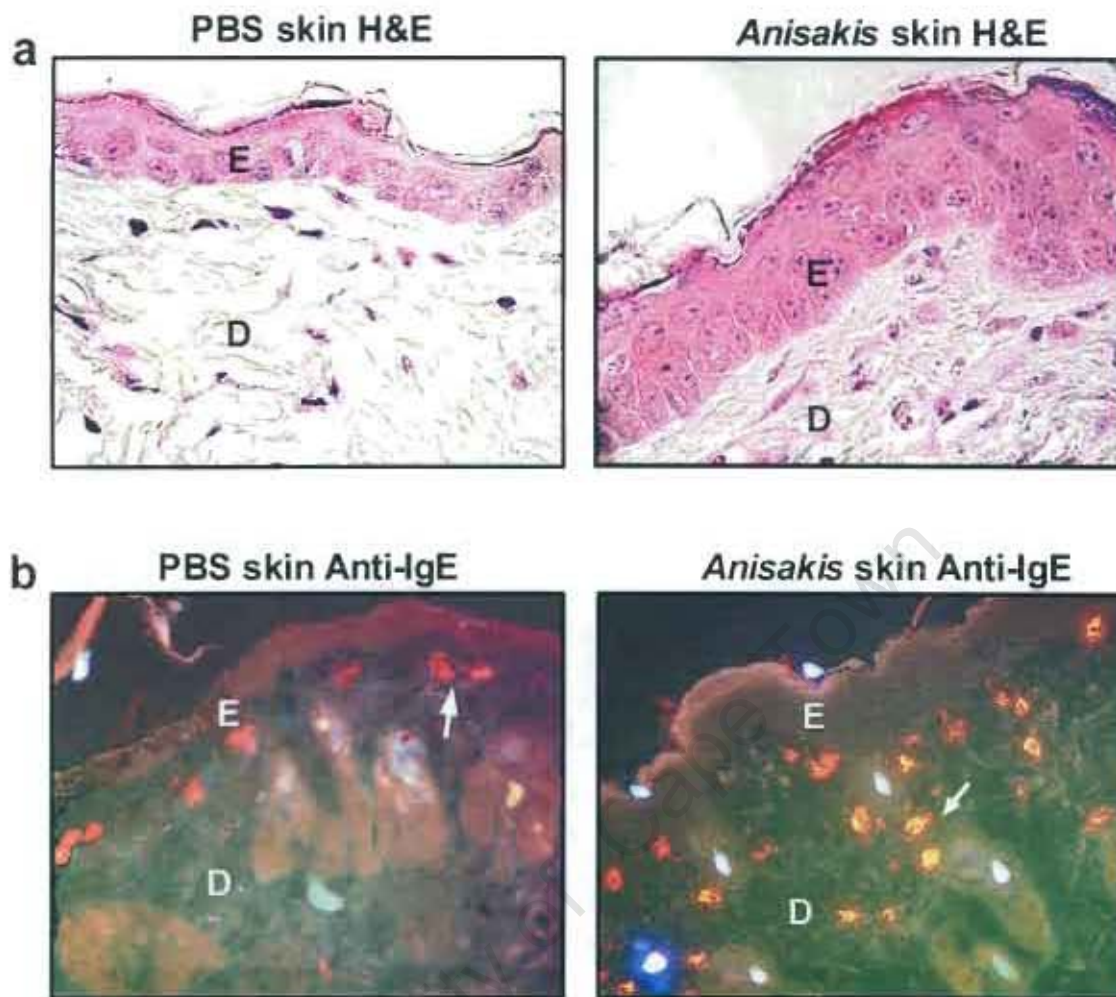


Figure 4.4.2. Histological and immunohistochemical analysis of PBS- and *Anisakis*-exposed skin. **a)** Skin sections were stained with H&E. The epidermis (E) and dermis (D) are labelled. *Anisakis*-exposed skin shows epidermal hyperplasia. Photographs are representative of several experiments, n = 4-5. **b)** Skin sections were stained with anti-IgE. *Anisakis*-exposed skin showed increased IgE-bearing cells in the dermis. The arrow indicates an IgE-positive cell. E = epidermis, D = dermis. Photographs are representative of 2-3 experiments, n = 4-5.

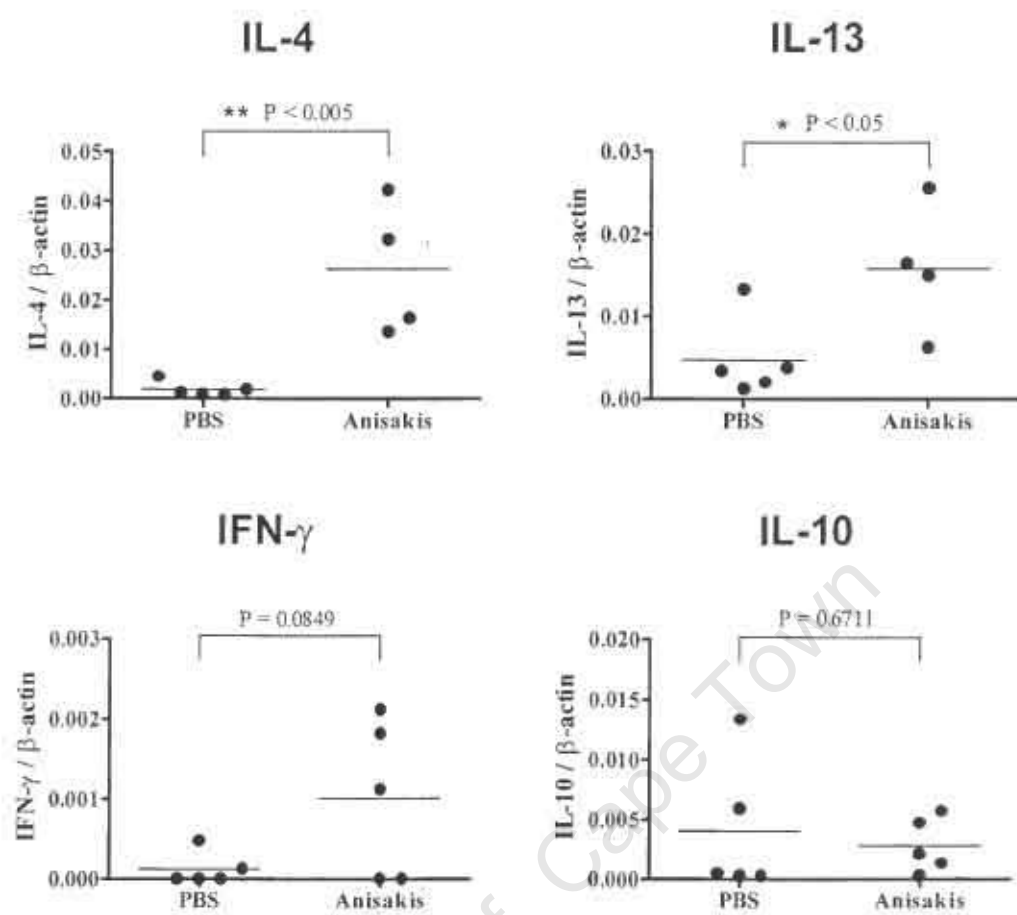


Figure 4.4.3. Levels of IL-4, IL-13, IFN- γ and IL-10 mRNA in skin biopsies from mice epicutaneously exposed to PBS or *Anisakis* extract. Levels were normalized to β -actin. Each circle represents skin from an individual mouse. P values are indicated. *, P < 0.05; **, P < 0.01.

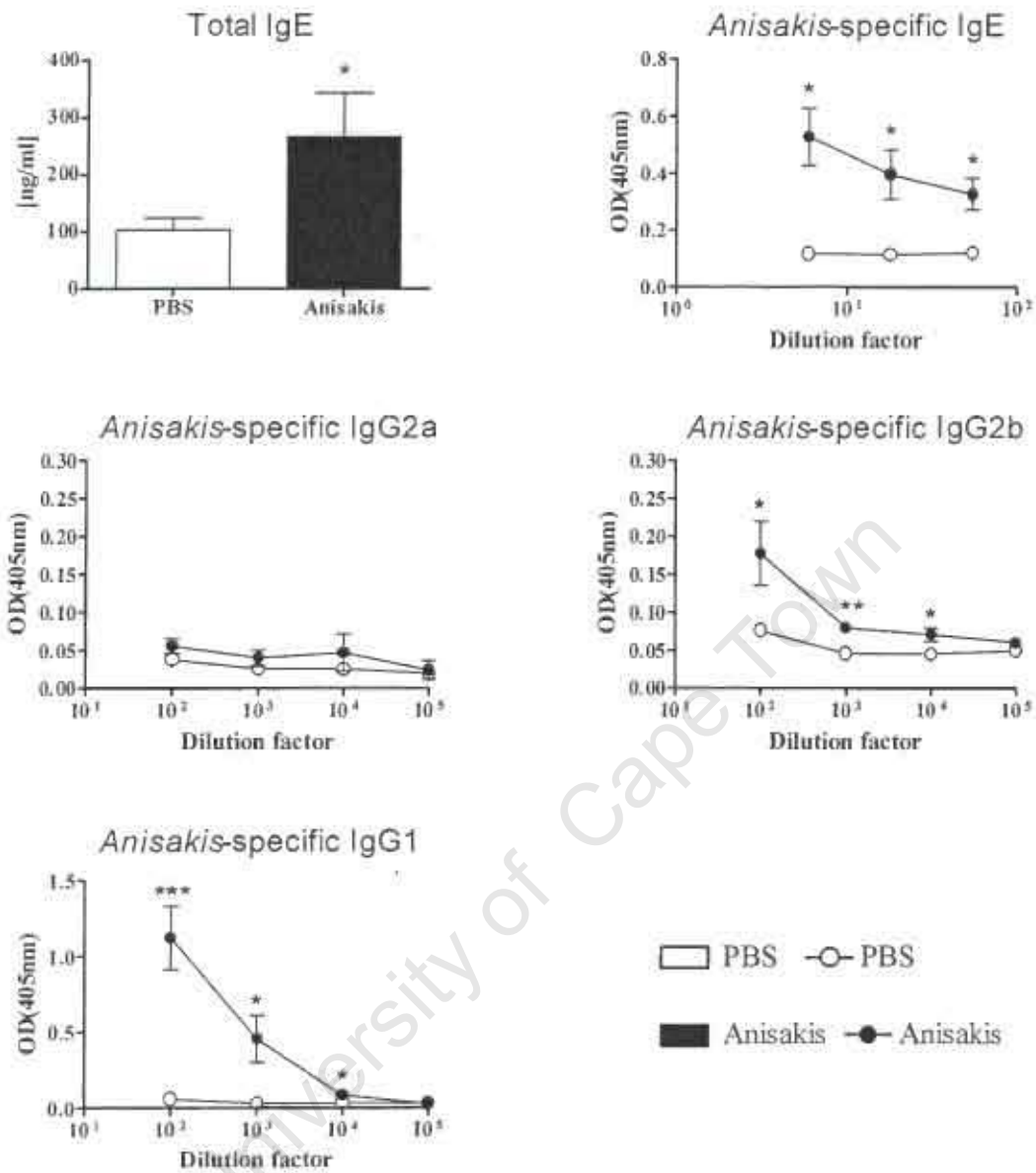


Figure 4.4.4. Total IgE and *Anisakis*-specific IgG1, IgG2a and IgG2b measured in the serum of mice epicutaneously exposed to PBS or *Anisakis* extract. Antibodies were measured by ELISA. The graphs are representative of three experiments, n = 3-5. Data presented as mean +/- SEM. Statistical significance between PBS- and *Anisakis*-sensitized mice is shown. *, P<0.05; **, P < 0.01; ***, P<0.001.

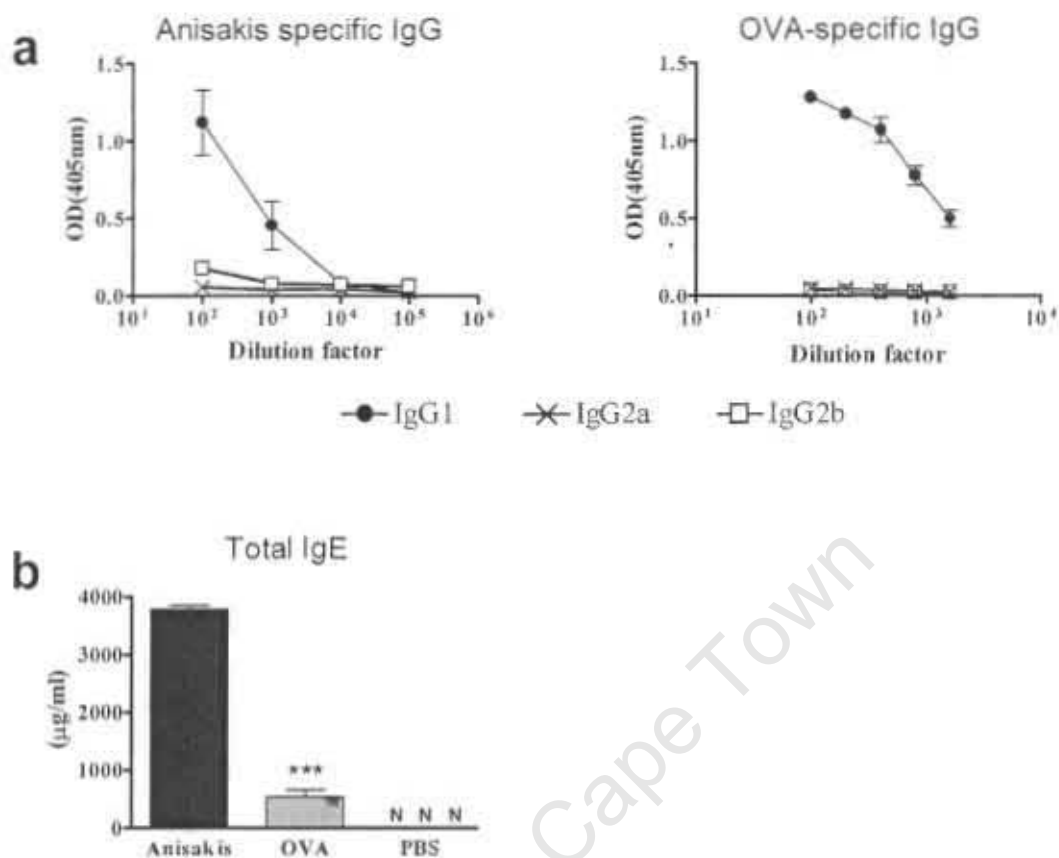


Figure 4.4.5. Comparison of IgG isotype and total IgE production in *Anisakis*- and OVA-sensitized mice. Antibodies were measured by ELISA. Values below the detection limit are indicated by N. Results are representative of two experiments, n = 3-4. Data presented as mean +/- SEM. Statistical significance between *Anisakis*-sensitized and OVA- or PBS-sensitized mice was calculated. ***, P<0.001.

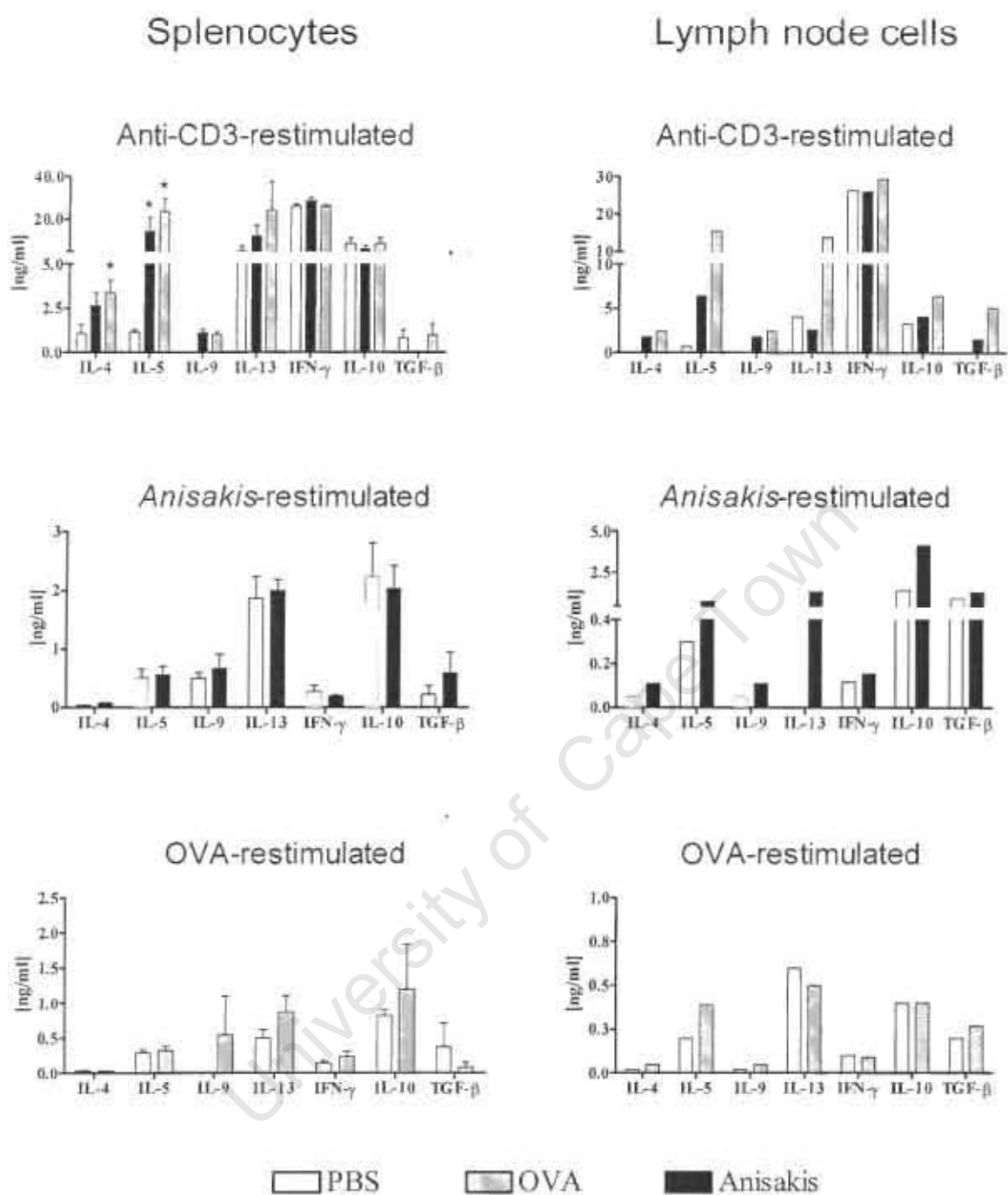


Figure 4.4.6. Cytokine production by splenocytes and skin-draining lymph node cells from epicutaneously sensitized mice. Cells from individual spleens (n = 4) and pooled lymph nodes (n = 4) were restimulated with anti-CD3 or sensitizing antigen (OVA/Anisakis extract). Supernatants were taken at 48 hours and cytokines were measured by ELISA. Data presented as mean +/- SEM. Statistical significance was assessed against cytokine production of cells from PBS control sensitized mice. *, P < 0.05.

lymph nodes from *Anisakis*-exposed mice with *Anisakis* extract showed increased production of IL-13 and IL-10 in particular, with a trend towards increased IL-5 production. However, OVA-restimulated lymph node cells from OVA-exposed mice did not show differences in cytokine production as compared to cells from PBS-exposed mice.

4.4.4 Intravenous challenge of epicutaneously sensitized mice results in anaphylaxis

After the removal of the third patch, mice exposed epicutaneously to PBS, OVA or *Anisakis* were challenged intravenously with 750 μ g of *Anisakis* or OVA to determine biologically relevant sensitization (Fig. 4.4.7). Mice in the PBS group did not react to either *Anisakis* or OVA challenge. No signs of anaphylaxis were observed and the rectal temperatures of the mice remained constant. In contrast, OVA or *Anisakis* sensitized mice showed signs of anaphylactic shock with reduced activity, piloerecti and temperature loss after challenge. Rectal temperatures of OVA-sensitized and challenged mice remained decreased for longer than rectal temperatures of *Anisakis*-sensitized and challenged mice.

4.4.5 Three applications of *Anisakis* extract were required to elicit anaphylaxis upon intravenous challenge

Intravenous challenges with *Anisakis* extract were performed after removal of the second patch or after removal of the third patch (Fig. 4.4.8). Upon challenge following removal of the second patch, mice showed vague signs of discontent and irritability, but did not have temperature loss or demonstrate signs of shock (Fig. 4.4.8a). However, upon challenge following removal of the third patch, mice showed signs of shock and temperature loss. This demonstrates that three patches were needed in order to sensitize mice for anaphylactic shock. This probably reflects an increase in antibody levels occurring between the second and third epicutaneous applications of *Anisakis* extract. However, intravenous antigen administration causes a decrease in free antibodies in the serum, so ELISA assays could not be used to accurately determine antibody levels. Restimulation of splenocytes with anti-CD3 showed that after the third patch, IL-4 and

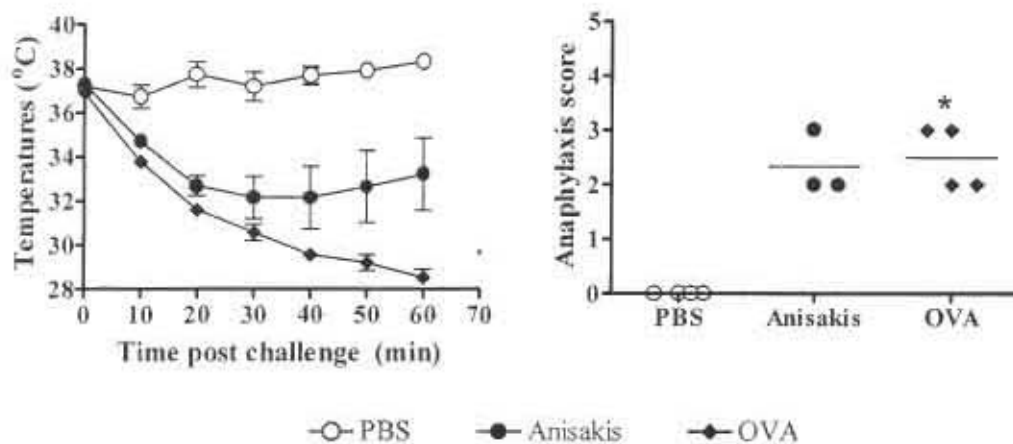


Figure 4.4.7. Anaphylactic responses following intravenous challenge of epicutaneously sensitized mice. Mice ($n = 3-4$) were epicutaneously exposed to PBS, *Anisakis* extract, or OVA at days 0-5, 11-15 and 21-25, then challenged intravenously on day 25 with *Anisakis* extract or OVA. Half of the PBS-exposed mice were challenged with *Anisakis* extract and the other half with OVA. No differences were seen between these two groups. Rectal temperatures were recorded for 1 hour following challenge. Reactions were rated using a scoring system (0, no reactions; 1, irritability, scratching and/or hypersensitivity to touch; 2, reduced activity and/or diarrhoea; 3, lying down, weakness and/or cyanosis; 4, lying on side, loss of grip, no activity upon prodding, loss of consciousness with or without convulsions; 5, death.) Data presented as mean \pm SEM.

IL-10 production were increased and IFN- γ production was decreased as compared responses in splenocytes from PBS controls and splenocytes taken after two patches, indicating a trend towards systemic Th2 skewing after repeated epicutaneous *Anisakis* extract exposures (Fig. 4.4.8b).

4.4.6 Local pathology induced by epicutaneous *Anisakis* exposure is primarily dependent on IL-13 and the IL-4R α

Mice deficient in IL-4, IL-13, IL-4/IL-13 or the IL-4R α were exposed to three epicutaneous applications of *Anisakis* antigen. IL-4 $-/-$ mice showed similar epidermal hyperplasia and dermal thickening to wildtype mice, and also exhibited a strong infiltration of immune cells (Fig. 4.4.9). However, very few infiltrating eosinophils were visible in tissue sections from this strain.

IL-13 $-/-$ and IL-4/IL-13 $-/-$ mice had dramatically reduced epidermal hyperplasia and dermal thickening. In addition, infiltration of immune cells into the dermis was strongly reduced in IL-13 $-/-$ and IL-4/IL-13 $-/-$ mice. IL-4R α $-/-$ mice also had reduced epidermal hyperplasia, dermal thickening and infiltration of immune cells as compared to wildtype mice. However, a small degree of inflammation remained, contrary to IL-13 $-/-$ mice, in which virtually no inflammation was present at all. Together the data indicate a primary role for IL-13 in local skin pathology due to epicutaneous application of *Anisakis* extract.

4.4.7 Differences in isotype antibody production in IL-4 $-/-$, IL-13 $-/-$ and IL-4R α $-/-$ mice

Unlike wildtype mice in which *Anisakis*-specific IgG1 and total and *Anisakis*-specific IgE were raised, IL-4 $-/-$ mice had undetectable levels of both specific and total IgE and only low *Anisakis*-specific IgG1 levels (Fig. 4.4.10 and Fig. 4.4.11). *Anisakis*-specific IgG2a levels were raised, indicating skewing towards a type 1 response. IL-13 $-/-$ mice in contrast maintained production of total IgE, though at lower levels compared to wildtype

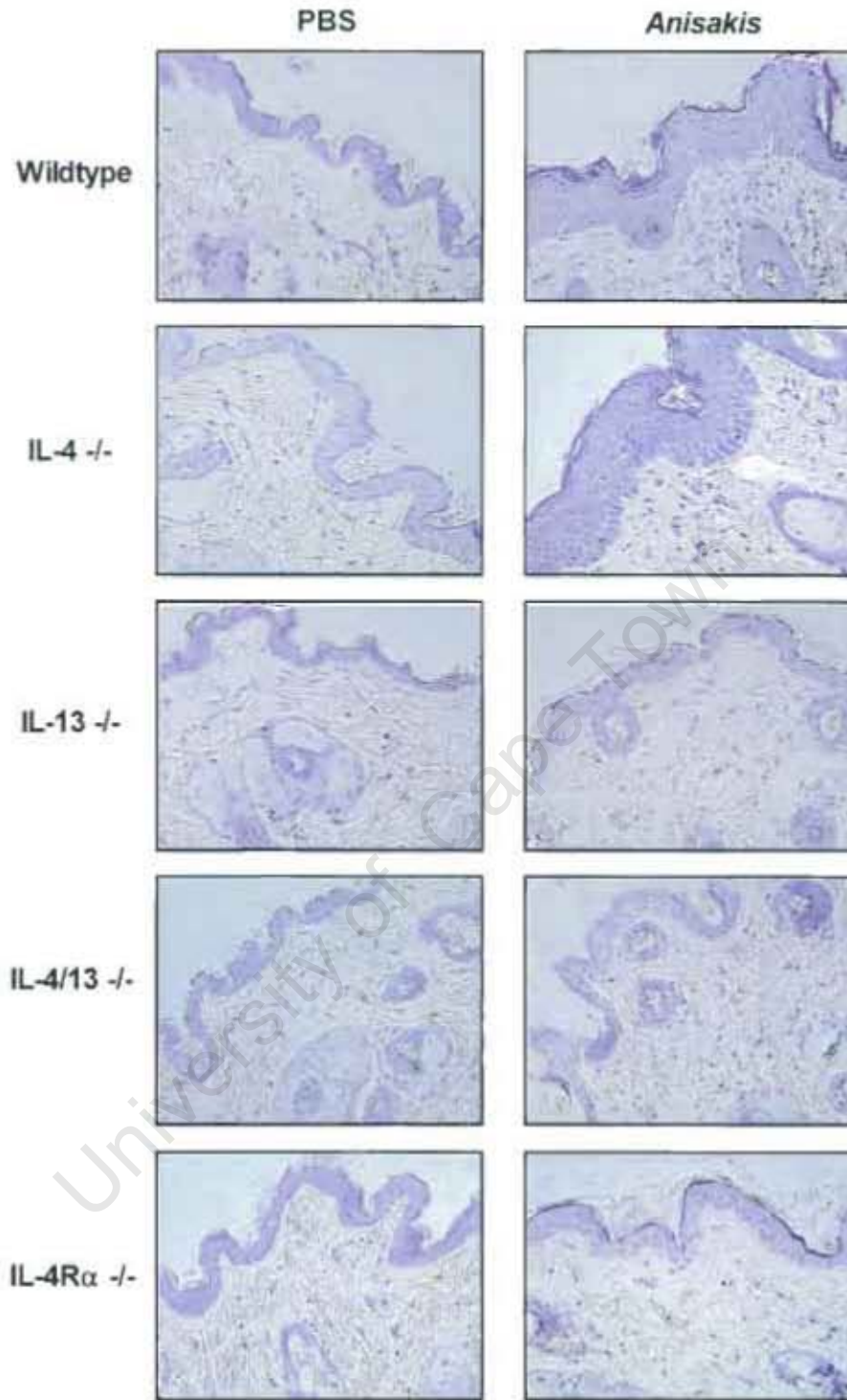


Figure 4.4.9. Histological features of wildtype and gene deficient mouse skin epicutaneously exposed to PBS or *Anisakis* extract. Sections were stained with H&E. Photographs were taken at 400x. The photographs are representative of 2 experiments, n = 4.

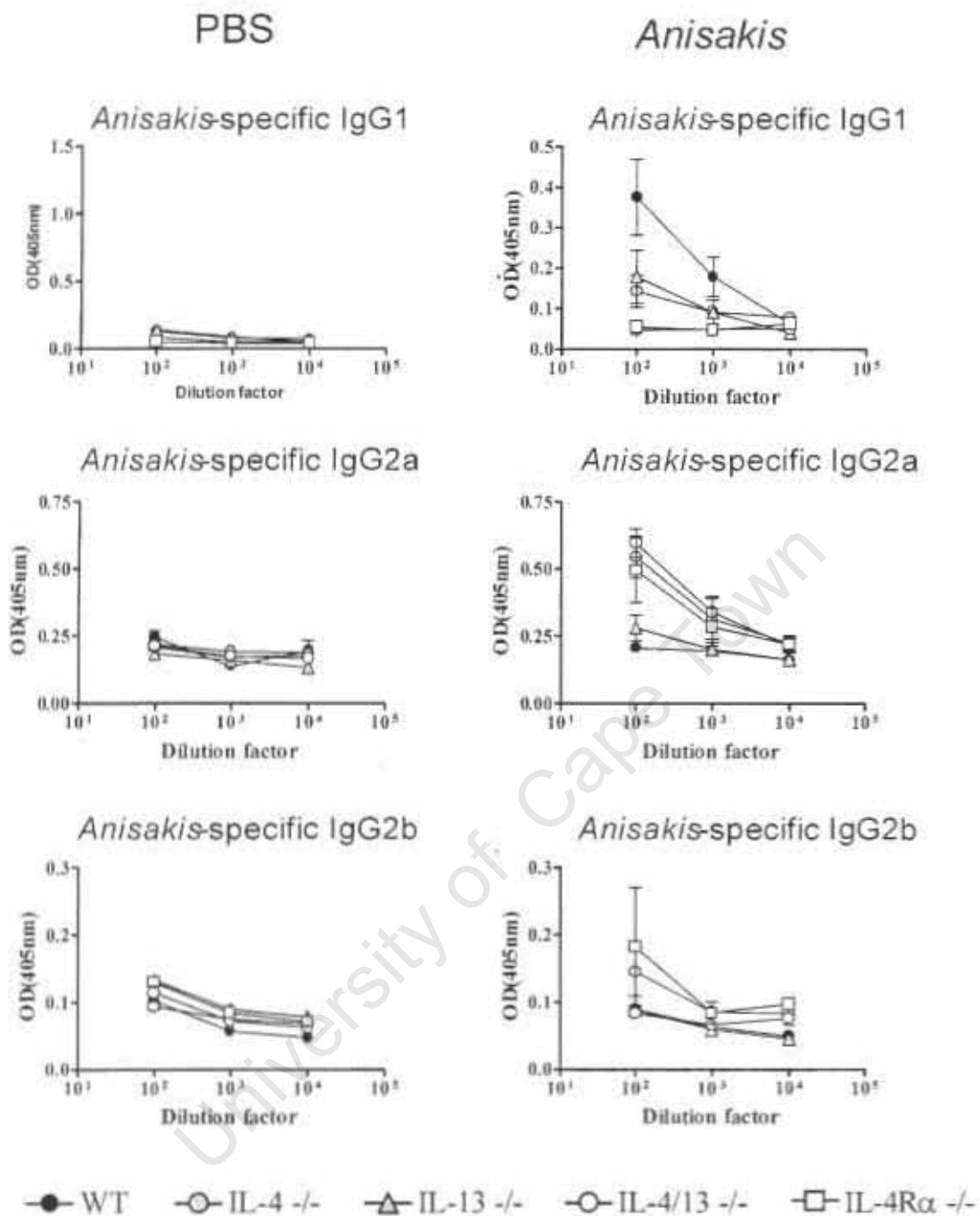


Figure 4.4.10. Serum IgG isotypes in wildtype and gene deficient mice. Mice were epicutaneously exposed to PBS or *Anisakis* extract (n = 4), and *Anisakis*-specific IgG1, IgG2a and IgG2b were measured by ELISA. Data presented as mean +/- SEM.

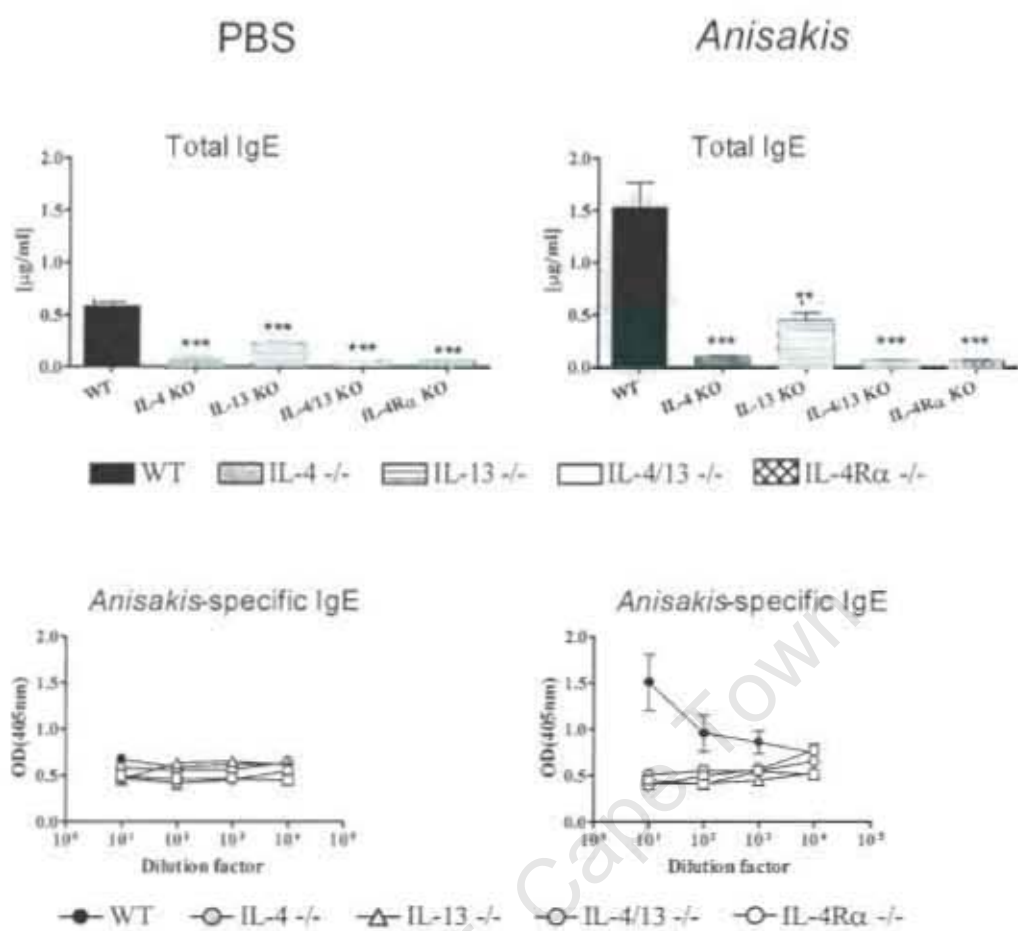


Figure 4.4.11. Total and *Anisakis*-specific IgE in wildtype and gene deficient mice. Mice were epicutaneously exposed to PBS or *Anisakis* extract (n = 4), and total and *Anisakis*-specific IgE were measured by ELISA. Data presented as mean +/- SEM. Statistical significance was calculated between wildtypes and other strains. **, P<0.01; ***, P < 0.001.

mice, although specific IgE was undetectable in the ELISA assay used. Levels of *Anisakis*-specific IgG1 and IgG2a were decreased in IL-13 $-/-$ mice. IL-4/13 $-/-$ and IL-4R α $-/-$ mice had low levels of IgG1 and undetectable levels of IgE, but raised IgG2a levels, indicating type 1 isotype skewing. Overall the results indicate an important role for IL-4 in controlling antibody isotype switching in this model.

4.4.8 Differences in Th1/Th2 profiles in IL-4 $-/-$, IL-13 $-/-$ and IL-4R α $-/-$ mice

Cytokine production by splenocytes restimulated with anti-CD3 was compared between strains (Fig. 4.4.12). Splenocytes from wildtype controls produced the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 as well as IL-10 and low levels of IFN- γ . Splenocytes from IL-4 $-/-$ mice did not produce IL-9, but maintained the production of IL-5 and IL-13, albeit at lower levels than in wildtype mice. IL-13 $-/-$ and IL-4/13 $-/-$ mice did not show production of IL-4, IL-9 or IL-13, but maintained production of IL-5, at lower levels than in wildtype mice. All three knockout strains (IL-4 $-/-$, IL-13 $-/-$ and IL-4/13 $-/-$) showed a trend towards decreased production of IL-10 and increased production of IFN- γ compared to wildtype mice, indicating some degree of shift towards a Th1 profile. The trend towards decreased production of IL-10 was also observed in splenocytes from these strains that were restimulated with *Anisakis* extract.

4.4.9 IL-4 and the IL-4R α are important in anaphylaxis following epicutaneous sensitization to *Anisakis*

After intravenous challenge of epicutaneously sensitized mice, IL-4 $-/-$ mice had reduced temperature loss and signs of shock, in contrast to IL-13 $-/-$ mice, which behaved the same as wildtypes (Fig. 4.4.13a,b). IL-4/IL-13 $-/-$ mice and IL-4R α $-/-$ mice were completely resistant to anaphylaxis in this model. In correlation with anaphylactic responses, only wildtype and IL-13 $-/-$ mice had raised levels of MMCP-1 after challenge (Fig. 4.4.13c). Wildtype and IL-13 $-/-$ mice were also the only strains in which IgE was raised (Fig. 4.4.11). This may suggest a correlation between IgE, mast cell degranulation

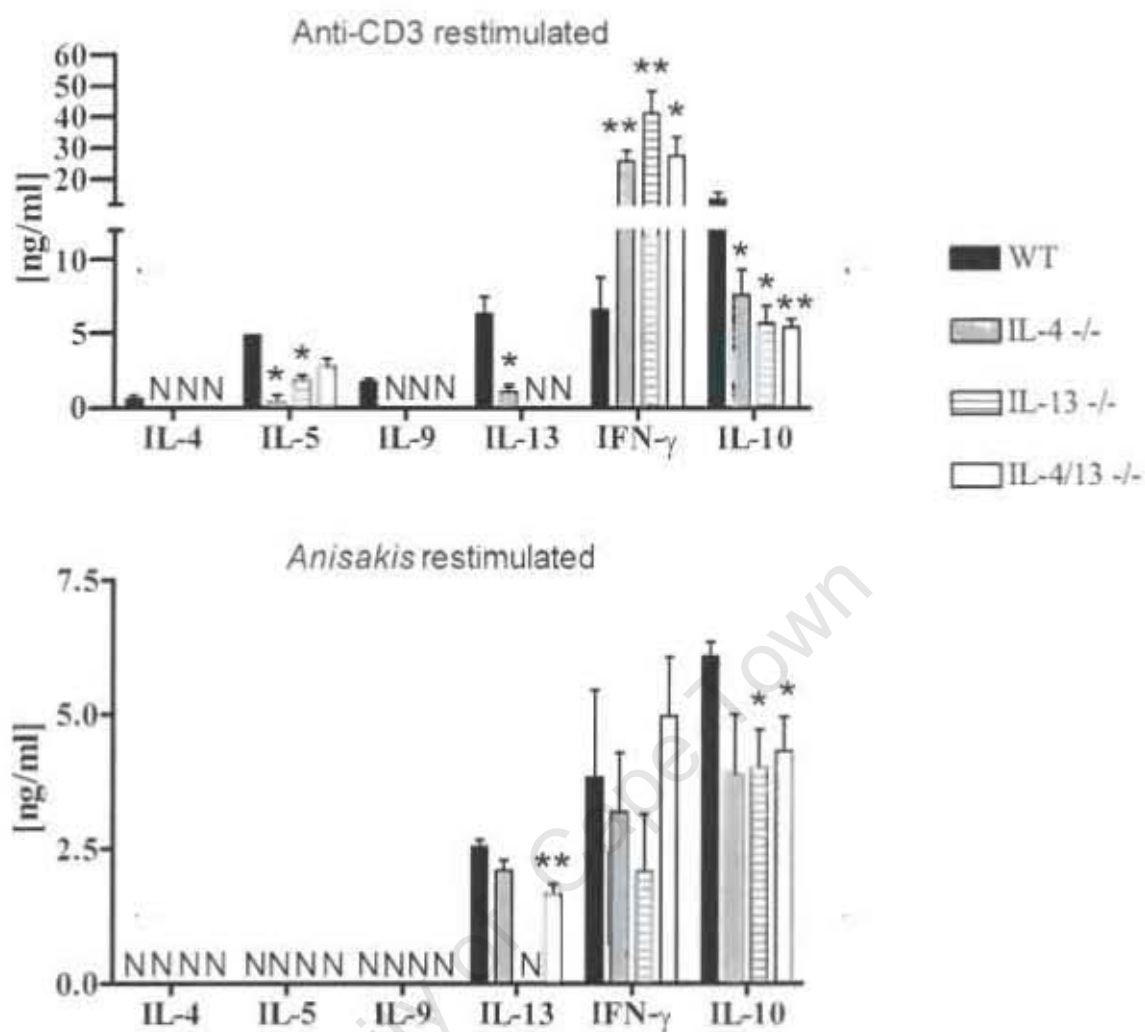


Figure 4.4.12. Cytokine production by splenocytes from epicutaneously sensitized wildtype and gene deficient mice. Cells from individual spleens ($n = 4$) were restimulated with anti-CD3 or *Anisakis* extract. Supernatants were taken at 48 hours and cytokines were measured by ELISA. Data presented as mean \pm SEM. Statistical significance was assessed between wildtypes and other strains. *, $P < 0.05$; **, $P < 0.01$; N, undetectable.

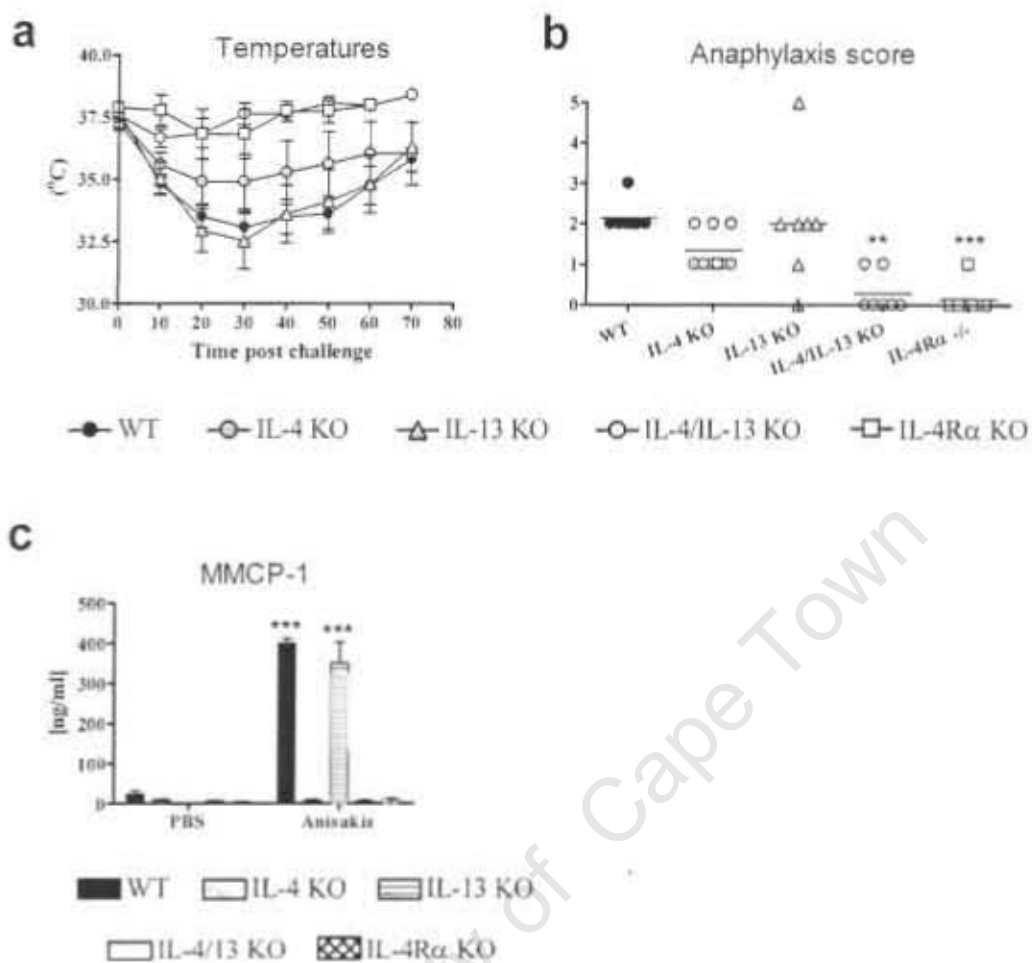


Figure 4.4.13. Anaphylactic responses following intravenous challenge of epicutaneously sensitized wildtype and gene deficient mice. Mice ($n = 7-9$, combined results of two experiments) were epicutaneously exposed to PBS or *Anisakis* extract at days 0-5, 11-15 and 21-25, then challenged intravenously on day 25 with *Anisakis* extract. a) Rectal temperatures were recorded for 70 minutes following challenge. PBS-exposed mice did not show temperature loss in response to *Anisakis* challenge (data not shown). Data presented as mean \pm SEM. b) Reactions were rated using a scoring system (0, no reactions; 1, irritability, scratching and/or hypersensitivity to touch; 2, reduced activity, pilar erecti and/or diarrhoea; 3, lying flat but upright, weakness and/or cyanosis; 4, lying on side, loss of grip, no activity upon prodding, loss of consciousness with or without convulsions; 5, death.). c) Serum MMCP-1 levels after challenge were measured by ELISA. Statistical significance was assessed between PBS- and *Anisakis*-sensitized mice. Data presented as mean \pm SEM. **, $P < 0.01$; ***, $P < 0.001$.

and the signs of anaphylaxis. As anaphylaxis was only partially reduced in IL-4 $-/-$ mice, other mechanisms such as IgG-mediated anaphylaxis may also play a role here. This would explain why IL-4R α $-/-$ mice, deficient in both IgE and IgG antibodies, are resistant to anaphylaxis in this model.

4.4.10 Anaphylaxis is partially abrogated in anti-IgE or anti-Fc γ RII/III treated mice

Mice were treated with anti-IgE, anti-Fc γ RII/III or control antibody prior to intravenous challenge in order to determine the roles of IgE and IgG in anaphylaxis following epicutaneous sensitization with *Anisakis* extract. Anti-IgE treatment effectively removed IgE from the serum and did not significantly affect IgG1 levels (it has been shown IgG1 is the anaphylactic IgG isotype in mice)^{74, 75} (Fig. 4.4.14a). Anti-Fc γ RII/III treatment did not affect IgE levels, but reduced measured IgG1 levels for reasons unknown. It was anticipated that intravenous antigen administration would decrease the levels of free antibody in the serum, but there is no obvious reason why this effect should be greater in one group than in the others. As the purpose of anti-Fc γ RII/III treatment was to block IgG-mediated anaphylaxis by binding to the Fc γ RII/III, the decrease in IgG1 in anti-Fc γ RII/III treated mice should not affect the interpretation of the results.

After intravenous challenge, both anti-IgE and anti-Fc γ RII/III treated mice exhibited anaphylactic reactions, with a slight but significant decrease in temperature loss compared to control treated mice (Fig. 4.4.14b). This suggests that either IgE or IgG can mediate anaphylaxis following epicutaneous sensitization, although IgG appears to play the more significant role. Unfortunately there was not enough depleting antibody left to repeat this experiment with the addition of a group treated with both anti-IgE and anti-Fc γ RII/III in order to confirm this hypothesis. MMCP-1 levels were raised in all three groups after challenge, suggesting that both IgE and IgG-mediated mast cell degranulation are possible in this model. Anti-ckit treatment at days 18 and 20 led to a reduction in serum MMCP-1 levels after challenge (Fig. 4.4.14c), indicating partial mast cell depletion, and was associated with decreased temperature loss after challenge and a quicker recovery from anaphylaxis (Fig. 4.4.15). This indicates that mast cell-mediated

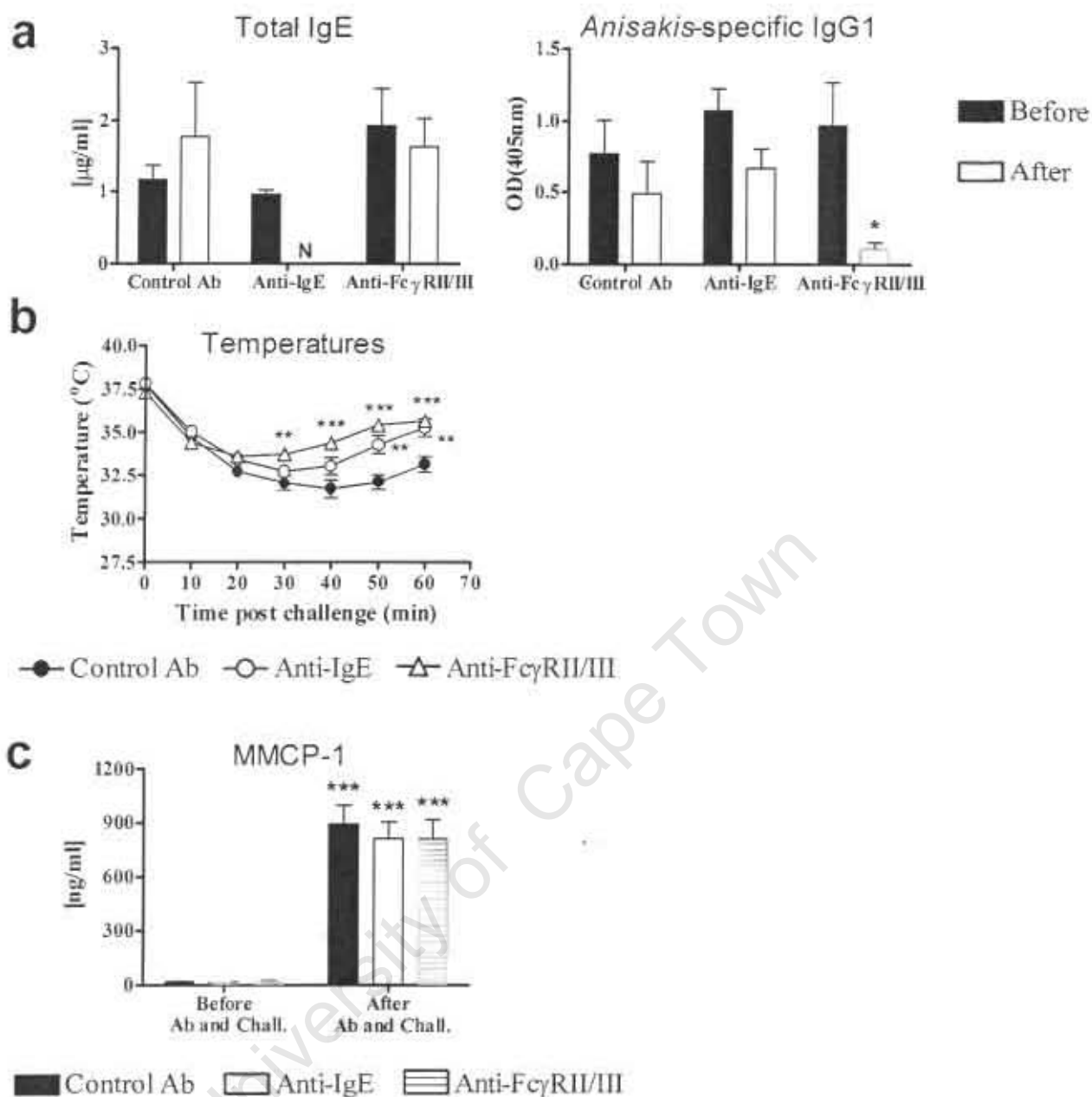


Figure 4.4.14. Anaphylactic responses following treatment with anti-IgE, anti-Fc γ RII/III or control antibody. Mice ($n = 7-8$, combined results of two experiments) were epicutaneously exposed to *Anisakis* extract at days 0-5, 11-15 and 21-25. Depleting antibody was administered on day 24, and mice were challenged i.v. with *Anisakis* extract on day 25. Results are expressed as mean \pm SEM. **a)** Total IgE and *Anisakis*-specific IgG1 were measured by ELISA. Before and after values were compared for statistical significance. **b)** Rectal temperatures were recorded for 1 hour after challenge. Statistical significance was assessed between control-treated mice and mice treated with a neutralizing antibody. **c)** MMCP-1 levels were measured in serum taken on day 24 and on day 25 after challenge. Before and after values were compared for statistical significance. $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

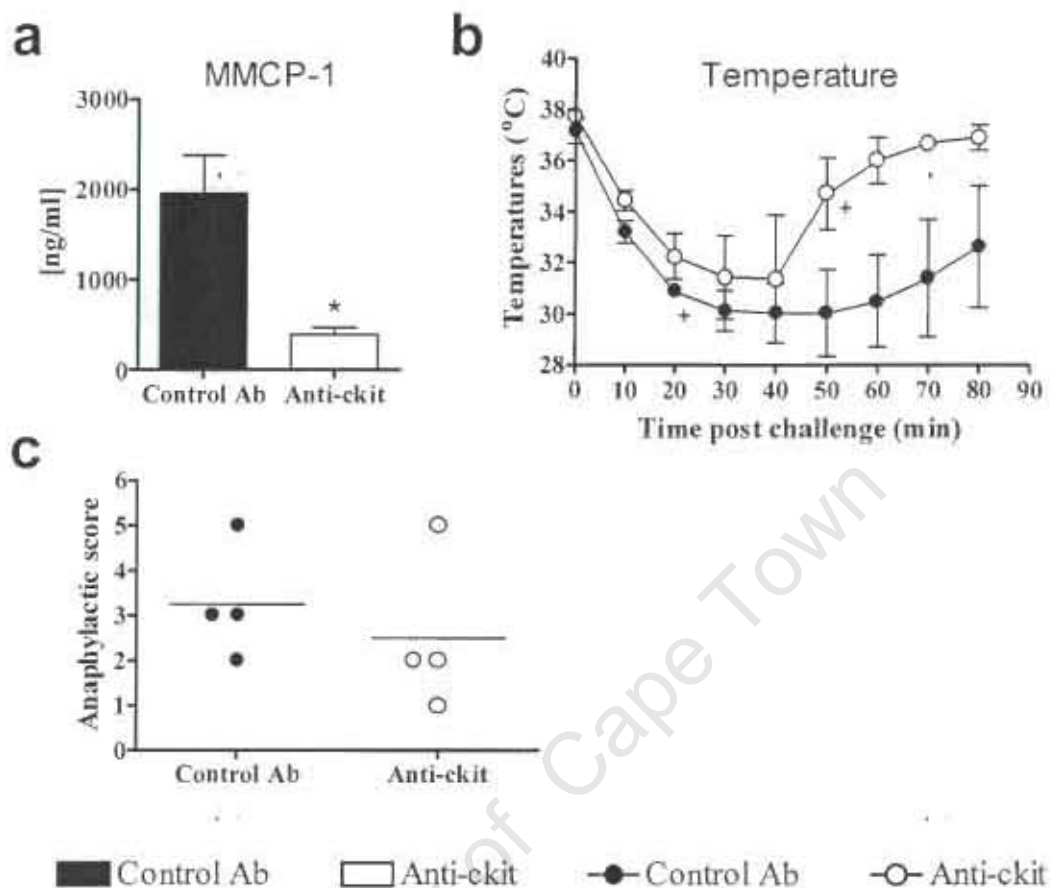


Figure 4.4.15. The effect of mast cell depletion on anaphylactic responses in epicutaneously sensitized mice. Mice ($n = 4$) were epicutaneously exposed to *Anisakis* extract at days 0-5, 11-15 and 21-25. Depleting antibody was administered on days 22 and 24, and mice were challenged intravenously with *Anisakis* extract on day 25. Data presented as mean \pm SEM. **a)** Mast cell depletion was assessed by measuring MMCP-1 levels in the serum after intravenous challenge. Statistical significance was assessed between control and anti-ckit treated mice. **b)** Rectal temperatures were recorded for 1 hour after challenge. **c)** Reactions were rated using a scoring system (0, no reactions; 1, irritability, scratching and/or hypersensitivity to touch; 2, reduced activity, pilar erecti and/or diarrhoea; 3, lying flat but upright, weakness and/or cyanosis; 4, lying on side, loss of grip, no activity upon prodding, loss of consciousness with or without convulsions; 5, death.). *, $P < 0.05$; +, death.

mechanisms must play at least a partial role in anaphylaxis due to epicutaneous sensitization.

4.4.11 The role of IL-4R α expression on CD4⁺ T cells and macrophages/neutrophils in local skin pathology following epicutaneous application of *Anisakis* extract

The experimental data shown above demonstrates that the IL-4R α plays an important role in local skin pathology, systemic sensitization and anaphylactic reactions following epicutaneous application of *Anisakis* extract. The availability of cell-specific IL-4R α $-/-$ mice enabled a more thorough dissection of mechanisms involved in epicutaneous sensitization by allowing for determination of the necessity for IL-4R α on various cell types.

As in previous experiments, epicutaneous application of *Anisakis* extract caused localized dermatitis in wildtype mice consisting of epidermal hyperplasia, dermal thickening and inflammatory cell infiltration (Fig. 4.4.16). This was reduced in IL-4R α $-/-$ mice. A minority of IL-4R α $-/-$ mice developed epidermal hyperplasia almost comparable to that seen in wildtype mice (approximately 25%, data not shown), but did not show increased infiltration of inflammatory cells compared to PBS controls.

Like wildtype mice, after epicutaneous application of *Anisakis* extract, CD4⁺ T-cell specific IL-4R α $-/-$ mice developed dermatitis characterized by epidermal hyperplasia, dermal thickening and infiltration of immune cells (Fig. 4.4.16). On average, epidermal hyperplasia in CD4⁺ T-cell specific IL-4R α $-/-$ mice appeared the same as in wildtype mice, but occasionally epidermal hyperplasia was strongly exacerbated (data not shown). In these cases, parakeratosis was enhanced. Macrophage/neutrophil specific IL-4R α $-/-$ mice also developed dermatitis similar to that of wildtype mice. *Anisakis*-exposed mice did not show differences in pathology compared to OVA-exposed mice in any of the strains tested (data not shown).

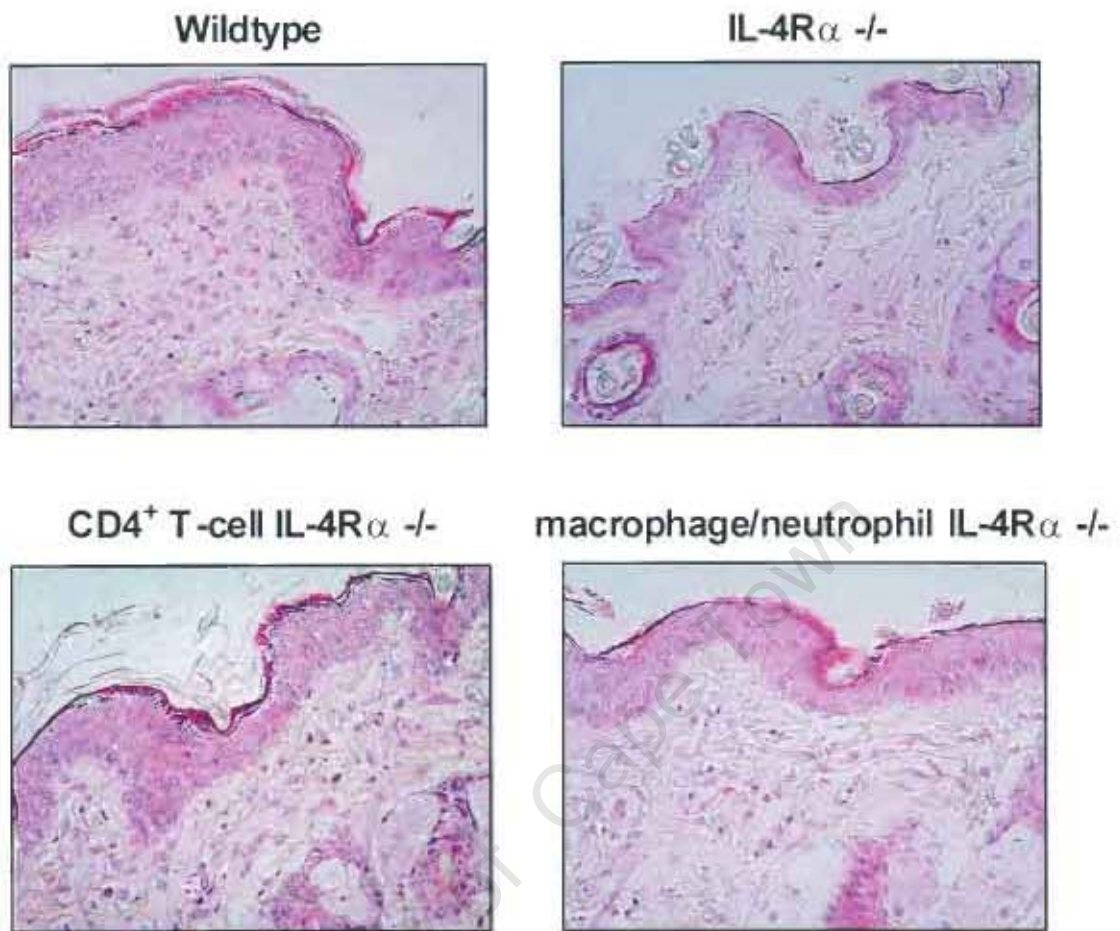


Figure 4.4.16. Histological features of skin sites epicutaneously exposed to *Anisakis* extract in wildtype, IL-4R α $-/-$, CD4 $^{+}$ T-cell specific IL-4R α $-/-$ and macrophage/neutrophil specific IL-4R α $-/-$ mice. Sections were stained with H&E. Photographs were taken at 400x. The photographs are representative of 3 experiments, n = 4-5.

4.4.12 The role of IL-4R α expression on CD4⁺ T cells and macrophages/neutrophils in systemic sensitization following epicutaneous application of *Anisakis* extract

Antibody isotypes were measured in the serum following epicutaneous *Anisakis* extract application (Fig. 4.4.17). Wildtype mice showed a type 2 isotype profile, with high levels of total IgE and *Anisakis*-specific IgG1, but only very low levels of *Anisakis*-specific IgG2a and IgG2b. Macrophage/neutrophil specific IL-4R α $-/-$ mice also showed type 2 skewing, with high levels of total IgE, moderate amounts of *Anisakis*-specific IgG1 and low levels of *Anisakis*-specific IgG2a and IgG2b. In contrast, CD4⁺ T-cell specific cell IL-4R α $-/-$ mice showed a mixed type 1/type 2 antibody profile, with comparatively high levels of total IgE, and *Anisakis*-specific IgG1 and IgG2a, but levels of *Anisakis*-specific IgG2b comparable to wildtypes. Complete IL-4R α $-/-$ showed type 1 antibody skewing, with high levels of *Anisakis*-specific IgG2a and IgG2b, but virtually no *Anisakis*-specific IgG1 and total IgE. This indicates that expression of IL-4R α on CD4⁺ T cells plays a role in inhibiting type 1 antibody isotype switching following epicutaneous sensitization, probably by mediating the differentiation of naïve T cells into Th2 cells producing IL-4 and IL-13.

4.4.13 The role of IL-4R α expression on CD4⁺ T cells and macrophages/neutrophils in Th1/Th2 skewing following epicutaneous application of *Anisakis* extract

Restimulated splenocytes from CD4⁺ T cells produce a mixed Th1/Th2 response, with production of both IFN- γ and production of Th2 effector cytokines (IL-5, IL-9 and IL-13)(Fig. 4.4.18). IFN- γ production tended to be raised in CD4⁺ T-cell specific IL-4R α $-/-$ mice, while Th2 cytokines tended to be lower. Restimulated splenocytes from macrophage/neutrophil IL-4R α $-/-$ mice produced significantly lower levels of IL-5, IL-9 and TGF- β than wildtype mice, whereas IL-4, IL-13, IL-10 and IFN- γ production was not significantly affected.

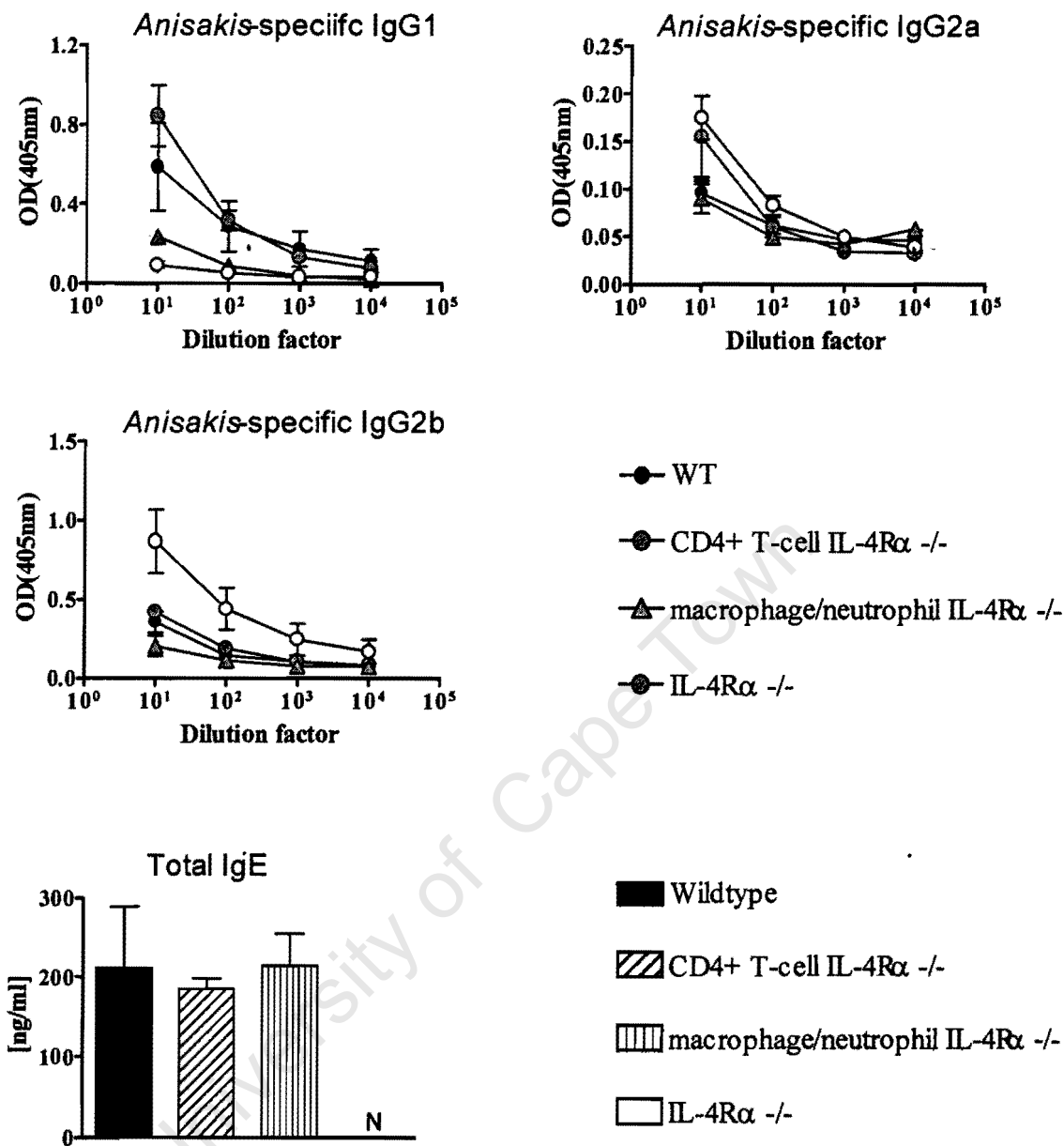


Figure 4.4.17. Antibody production in wildtype, IL-4Rα -/- and cell specific IL-4Rα -/- mice after epicutaneous sensitization. Mice were epicutaneously exposed to *Anisakis* extract (n = 3-5), and *Anisakis*-specific IgG1, IgG2a and IgG2b were measured by ELISA. Data are representative of two experiments. Data presented as mean +/- SEM.

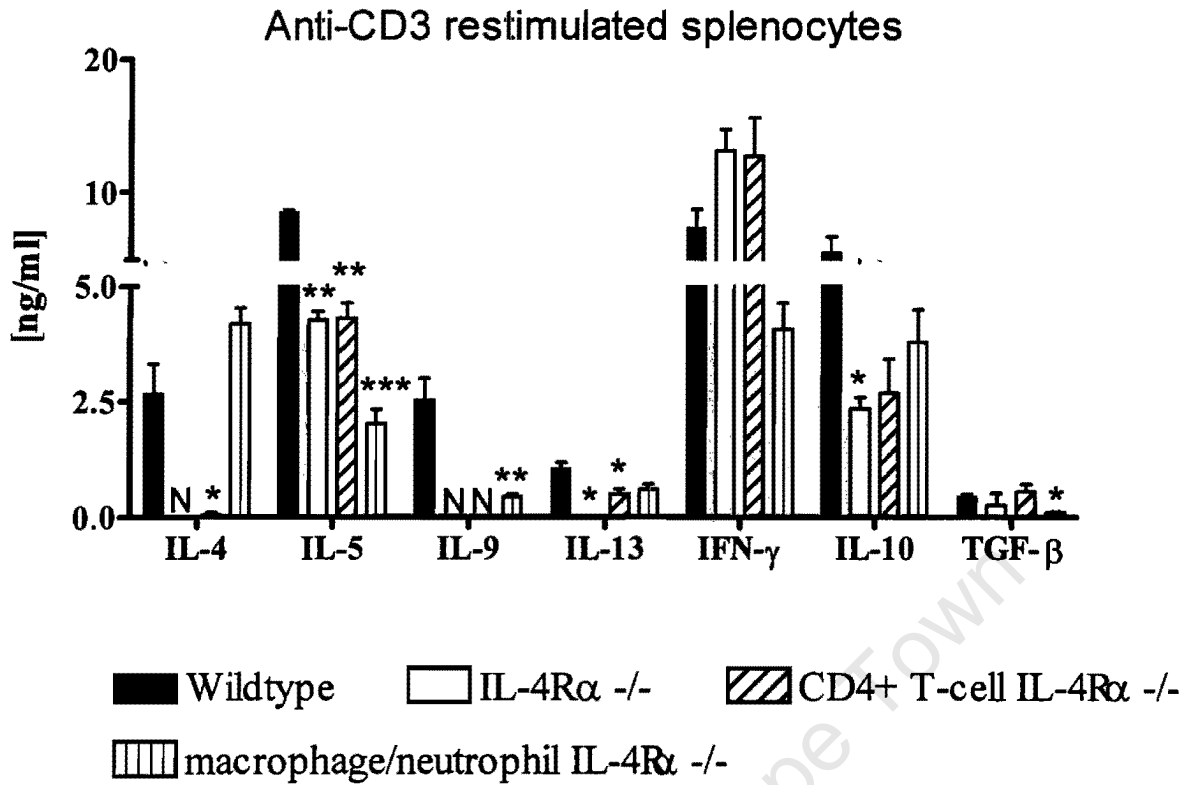


Figure 4.4.18. Cytokine production by splenocytes from epicutaneously sensitized wildtype, IL-4Rα -/- and cell specific IL-4Rα -/- mice. Cells from individual spleens (n = 4) were restimulated with anti-CD3. Supernatants were taken at 48 hours and cytokines were measured by ELISA. Data presented as mean +/- SEM. Statistical significance was assessed between wildtype mice and other strains. *, P < 0.05; **, P < 0.01; ***, P < 0.001; N, undetectable.

4.4.14 The role of IL-4R α expression on CD4⁺ T cells and macrophages/neutrophils in anaphylaxis following epicutaneous sensitization with *Anisakis* extract

Intravenous challenge with *Anisakis* extract was given to mice sensitized epicutaneously with *Anisakis* extract. After the challenge, CD4⁺ T-cell specific IL-4R α $-/-$ mice developed anaphylaxis, but responses were significantly attenuated compared to those in wildtype mice (Fig. 4.4.19a,b). This was reflected by a decrease in temperature loss. MMCP-1 levels were not significantly lowered (Fig. 4.4.19c).

Following intravenous challenge of *Anisakis*-sensitized mice with *Anisakis* extract, macrophage/neutrophil specific IL-4R α $-/-$ mice developed anaphylaxis to the same degree as wildtype mice. Therefore IL-4R α expression on macrophages and neutrophils, and accordingly the presence of alternative macrophages, does not play a major role in the development of systemic sensitization and allergic reactions following epicutaneous antigen application.

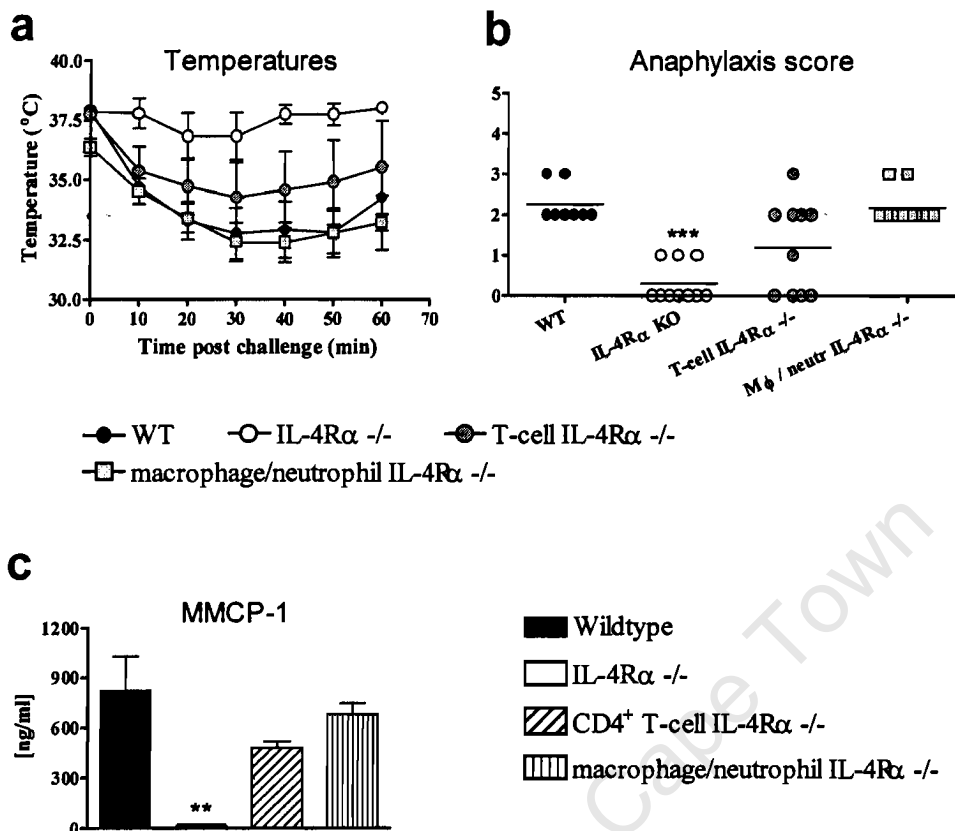


Figure 4.4.19 Anaphylactic responses following intravenous challenge of epicutaneously sensitized wildtype, IL-4Rα -/- and cell specific IL-4Rα -/- mice. Mice (n = 8-10, combined results of two experiments) were epicutaneously exposed to *Anisakis* extract at days 0-5, 11-15 and 21-25, then challenged intravenously on day 25 with *Anisakis* extract. Data presented as mean +/- SEM. **a)** Rectal temperatures were recorded for 1 hour following challenge. **b)** Reactions were rated using a scoring system (0, no reactions; 1, irritability, scratching and/or hypersensitivity to touch; 2, reduced activity, pilar erecti and/or diarrhoea; 3, lying flat but upright, weakness and/or cyanosis; 4, lying on side, loss of grip, no activity upon prodding, loss of consciousness with or without convulsions; 5, death). **c)** Serum MMCP-1 levels after challenge were measured by ELISA. Statistical significance was assessed between wildtype mice and other strains. *, P<0.05; **, P<0.01; ***, P<0.001.

4.5 DISCUSSION

The data presented in this chapter demonstrate for the first time that epicutaneous exposure to *Anisakis* extract can cause systemic sensitization in mice, and shows distinctive roles for the cytokines IL-4 and IL-13 during sensitization via the epicutaneous route and subsequent systemic allergic reactions.

Allergic skin diseases are associated with considerable morbidity, particularly when chronic¹⁸. Two populations at special risk of allergic skin disease are children born to atopic families, and people working in jobs that involve wet work, latex/rubber gloves, friction, repeated microtraumas or exposure to solvents or caustic materials. The former group is at an increased risk of developing eczema while the latter is at an increased risk of developing eczema, contact dermatitis and contact urticaria^{18, 76}. The present study uses an experimental mouse model to dissect the immunological mechanisms of eczema induced by *Anisakis* extract, a relevant occupational sensitizing agent. Allergy to *Anisakis* proteins in the working environment has been demonstrated in several studies^{5, 7, 8, 77, 78}. Reactions to *Anisakis* include protein contact dermatitis, conjunctivitis, asthma and rhinitis.

Wildtype BALB/c mice exposed to *Anisakis* extract developed epidermal hyperplasia, dermal thickening and infiltration of immune cells into the dermis. IgE-bearing cells were shown infiltrating the dermis of *Anisakis*-exposed mice, mimicking the response seen in patients with eczema^{32, 58}. The increased expression of IL-4 and IL-13 mRNA in *Anisakis* exposed mice is also in agreement with data from human eczema sufferers^{11, 38}. Taken together these data demonstrate the relevance of the mouse model to this study.

In order to establish the specific roles of IL-4, IL-13 and the IL-4R α in local skin inflammation, we utilized gene deficient mice. Mice deficient in IL-4 maintained skin inflammation, although the infiltrate contained fewer visible eosinophils, in agreement with previous studies⁶³. In contrast, in IL-13^{-/-} mice the skin pathology following *Anisakis* exposure was strikingly reduced, with few signs of epidermal hyperplasia or cellular infiltration. Pathology was similarly reduced in mice deficient in both IL-4 and

IL-13 (IL-4/13 $-/-$) or in mice deficient in the IL-4R α , through which both IL-4 and IL-13 signal. This suggests an important role for IL-13 signalling through the IL-4R α in mediating skin inflammation following epicutaneous exposure to *Anisakis* extract, and most likely other allergens as well. In all experiments where OVA sensitization was performed in parallel with *Anisakis* extract sensitization, there were no noticeable differences between responses to these two substances.

Having established the importance of the IL-4R α in *Anisakis*-induced skin inflammation, we used cell specific IL-4R α $-/-$ mice to determine the necessity for IL-4R α expression on CD4 $^+$ T cells, macrophages and neutrophils. It has been previously shown that a predominance of CD4 $^+$ T cells and macrophages exists in the cellular infiltrate of OVA-induced dermatitis in mice, and that $\alpha\beta^+$ TCR cells in particular are critical for skin inflammation and the generation of Th2 responses^{53, 63, 79}. In the current study, a similar predominance of macrophages and lymphocytes was observed in the cellular infiltrate. However, the results of experiments using CD4 $^+$ T cell specific IL-4R α $-/-$ mice (Lck^{cre}IL-4R α ^{-lox}) and macrophage/neutrophil specific IL-4R α $-/-$ mice (LysM^{Cre}-IL-4R α ^{-lox}) show that IL-4R α expression on CD4 $^+$ T cells, macrophages or neutrophils does not play a major role in regulating skin inflammation. CD4 $^+$ T-cell specific IL-4R α $-/-$ mice showed similar pathology to wildtype mice, despite having partially reduced Th2 responses. Macrophage/neutrophil specific IL-4R α $-/-$ mice also had similar epidermal hyperplasia to wildtype mice, indicating that IL-4R α expression on macrophages or neutrophils was not critical for acute skin inflammation. It is possible that alternative macrophages may play a more important role in chronic eczema, in which skin remodeling occurs³⁸.

The data from this model suggest two possibilities: 1) IL-4R α expression on another cell type is critical in local skin pathology, via IL-13 signalling. This may be a local skin cell such as an epithelial cell, keratinocyte, Langerhans cell or dermal dendritic cell. In this case, topical application of an IL-4R α antagonist or anti-IL-13 monoclonal antibody might be sufficient to reduce skin inflammation and might remove the necessity for

systemic treatment. Experimental models of asthma show that IL-13 has direct effects upon non-immune cells such as epithelial cells, endothelial cells, goblet cells and smooth muscle cells⁸⁰⁻⁸⁷. It is possible that IL-13 functions in a similar way to induce pathological changes in the skin.

2) Resistance to skin inflammation in IL-4R α -/- and IL-13 -/- mice is due to Th1 skewing and/or Th2 cytokine reduction in these strains. As far as this possibility is concerned, a recent study showed that IFN- γ -/- mice had decreased dermal thickening compared to wildtype mice, suggesting that IFN- γ enhances rather than abrogates skin hypertrophy in mice⁶³. IFN- γ is predominant in chronic eczematous lesions in humans⁸⁸ and is thought to exacerbate inflammation by causing keratinocyte apoptosis^{11, 89}. However, in human trials, recombinant IFN- γ treatment reduced skin inflammation in atopic eczema (but not nonatopic eczema)⁹⁰⁻⁹³. In another murine model, CpG DNA treatment increased IFN- γ production and reduced Th2 cytokine expression and the severity of eczema in 16/26 mice, but 10/26 mice had IFN- γ hyperproduction and showed exacerbation of lesions⁹⁴. Overall these studies indicate that the right amount of IFN- γ can be beneficial to atopic eczema, but that excessive IFN- γ may be detrimental.

In addition to local skin inflammation, mice epicutaneously exposed to *Anisakis* extract developed *Anisakis*-specific IgG1 and IgE antibodies, indicating systemic sensitization through the skin. Intravenous challenges were performed in order to certify that this sensitization was biologically relevant, and indeed, sensitized mice developed anaphylactic responses upon challenge. As sensitization via the skin may play a significant role in the development of subsequent allergic diseases, an understanding of the underlying mechanisms is crucial for the development of therapies that can intervene at this stage^{27, 51, 53}. This is of relevance for children showing the first signs of eczema and for workers demonstrating skin responses to occupational allergens.

Herrick et al investigated the role of IL-4 and IL-13 in airway hyperreactivity following epicutaneous sensitization with OVA, and concluded that IL-13 was the critical mediator in regulating Th2 activation after epicutaneous sensitization²⁷. In the current study, anaphylaxis after intravenous challenge was used to assess systemic sensitization to

Anisakis extract in various gene deficient and transgenic mice. The results showed that epicutaneously sensitized IL-13 $-/-$ mice did not have reduced anaphylactic responses after challenge. IL-13 $-/-$ mice produced both total IgE and *Anisakis*-specific IgG1, albeit at reduced levels, and had serum MMCP-1 levels equivalent to those of wildtype mice, indicating that mast cell degranulation occurred after challenge. In contrast, IL-4 $-/-$ mice showed a reduction in anaphylactic reactions including temperature loss. IL-4 $-/-$ mice produced approximately the same levels of IgG1 as IL-13 $-/-$ mice, but total IgE was strikingly decreased, as were MMCP-1 levels in the serum. Decreased MMCP-1 levels could result from reduced numbers of mast cells or reduced degranulation of available mast cells.

IL-4/13 $-/-$ and IL-4R α $-/-$ mice were completely protected against anaphylaxis. Both of these strains had very low levels of *Anisakis*-specific IgG1, total IgE and MMCP-1 present in the serum, while IgG2a levels were raised. Antibody depletion experiments with anti-IgE, anti-Fc γ RII/III and anti-ckit suggested that anaphylaxis in this model can be mediated by either IgE or IgG antibody, and is at least partially dependent on mast cell degranulation. As it has been demonstrated that IgG1 is the only anaphylactic IgG antibody in mice ⁷⁵, it is possible that the IL-4/13 $-/-$ and IL-4R α $-/-$ strains were protected because of a deficit of IgE and IgG1. Likewise, IL-4 $-/-$ mice may have been partially protected due to strongly reduced IgE synthesis, associated with strongly reduced MMCP-1 levels. IL-4 was more important for anaphylaxis than IL-13 in this model. However, IL-13 may have partially compensated for IL-4, since IL-4 $-/-$ mice had some degree of anaphylactic reactions, unlike IL-4/IL-13 $-/-$ and IL-4R α $-/-$ mice, which were completely protected. The data support an important role for IL-4R α in systemic sensitization via the epicutaneous route in mice.

Subsequently, the role of IL-4R α on CD4⁺ T cells and macrophages/neutrophils in anaphylaxis in epicutaneously sensitized mice was investigated. Macrophage/neutrophil specific IL-4R α $-/-$ mice reacted in the same way as wildtype mice to intravenous challenge, undergoing anaphylaxis with temperature loss. Anaphylaxis was partially reduced in CD4⁺ T-cell specific IL-4R α $-/-$ mice. The level of protection was not as

pronounced as the reduction in anaphylaxis in the intraperitoneal sensitization model described in chapter 3 of this thesis, highlighting mechanistic differences in different routes of sensitization, also seen by Herrick et al ⁶⁵. It is unclear why CD4⁺ T cell specific IL-4R α -/- mice experienced less severe anaphylaxis than wildtype mice. Total IgE and *Anisakis*-specific IgG1 levels were not reduced compared to wildtype mice. However, levels of the type 1 antibody IgG2a were raised. It is possible that IgG2a present in the serum may have bound a portion of the injected antigen, resulting in a decrease in free antigen available to bind IgE and/or IgG1. CD4⁺ T-cell specific IL-4R α -/- mice also demonstrated a trend towards decreased production of Th2 cytokines and increased production of IFN- γ compared to wildtype mice. In chapter 3 we demonstrated that the presence of IFN- γ was associated with a decrease in anaphylactic reactions.

The fact remains that neither CD4⁺ T-cell specific IL-4R α -/- mice or macrophage/neutrophil specific IL-4R α -/- mice were completely resistant to anaphylaxis as IL-4R α -/- mice were, nor had the strong reduction in Th2 cytokine production or IgE levels that IL-4R α -/- mice did. This suggests that IL-4R α on another cell type is important for induction of systemic sensitization, Th2 activation and IgE production via the skin, or that the overall phenotype resulting from the loss of IL-4R α on all cells is responsible for the resistance to anaphylaxis and Th2 activation. Future studies should investigate these hypotheses, as the outcome has relevance for therapies aimed at treatment of eczema and prevention of the atopic march.

In conclusion, the data demonstrate that epicutaneous exposure to *Anisakis* extract is a useful and relevant model of eczema in mice. The fact that *Anisakis* proteins appear capable of eliciting systemic sensitization via the skin highlights the importance of minimizing exposure to this and other allergens in fish processing workers. The Food and Agriculture Organization of the United Nations estimates that fishery and aquaculture production activities provided direct employment and revenue for 38 million people worldwide in 2002 ⁹⁵. In South Africa, the fishing industry employs approximately 90 000 workers ⁹⁶. An estimated 3-11% of workers involved in processing develop contact urticaria or protein contact dermatitis ⁷⁶. Occupational dermal exposure to fish or

fish juices occurs mainly as a result of unprotected handling of fish during processes such as heading, skinning, degutting, mincing, filleting, trimming, cooking, frying, fishmeal milling, bagging and freezing ^{76,97}. Various preventative measures can be taken to reduce exposure, including the use of cotton-lined gloves and plastic sleeves, the immediate treatment of puncture wounds and lacerations and the use of emollients and moisturizers to protect skin barrier function ⁹⁸. In order to intervene at an early stage to prevent exacerbation of symptoms and the atopic march, early subclinical biomarkers and target organ tests may be used for monitoring workers, such as eosinophil cationic protein, skin prick test, specific IgE or IgG, non-specific bronchial hyperresponsiveness or skin patch tests ⁷⁶.

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CONCLUSIONS

This study demonstrates that *Anisakis* proteins are allergenic and can cause ingestion-related allergic reactions, anaphylaxis and dermatitis in mice. IL-4, IL-13 and the common receptor subunit IL-4R α play important and distinct roles in these allergic diseases.

Mice deficient in IL-13 or the IL-4R α showed particularly enhanced inflammatory responses against intraperitoneally administered *Anisakis* larvae, compared to wildtype and IL-4 deficient mice. Wildtype mice demonstrated the least inflammation but the strongest memory responses, with elevated specific IgG1 and total and specific IgE. Live larvae predisposed wildtype mice to allergic reactions following subsequent ingestion of *Anisakis* proteins. Oral sensitization with *Anisakis* extract induced *Anisakis*-specific antibodies but was less effective at sensitizing for subsequent allergic reactions than live infection. Allergic reactions were reduced and abrogated in IL-4 $-/-$ and IL-4R α $-/-$ mice, respectively, suggesting important roles for IL-4, IL-13 and the IL-4R α in food allergy.

Anisakis proteins behaved similarly to ovalbumin in a mouse model of anaphylaxis. Mechanisms of anaphylaxis were explored using CD4 $^+$ T cell specific IL-4R α $-/-$ and macrophage/neutrophil specific IL-4R α $-/-$ mice. CD4 $^+$ T cell specific IL-4R α $-/-$ mice were protected against anaphylaxis by an IFN- γ dependent mechanism. Protection was associated with decreased degranulation of mast cells and may therefore be related to an *in vivo* inhibitory effect of IFN- γ on mast cell degranulation.

Epicutaneous application of *Anisakis* proteins in mice induced a localized dermatitis and led to biologically relevant systemic sensitization. Local skin pathology was dependent on IL-13, while IL-4 played an important role in systemic sensitization. Mice deficient in the IL-4R α were protected against both skin pathology and anaphylactic reactions following intravenous challenge of sensitized mice. Deletion of IL-4R α on CD4 $^+$ T cells did not reduce skin pathology, but increased resistance to anaphylactic reactions. In

contrast, deletion of IL-4R α on macrophages/neutrophils did not reduce anaphylaxis, but slightly decreased local skin pathology.

Based on the models used in this thesis, observations can be made about differences in the immune responses elicited by exposure to live *Anisakis*, *Anisakis* extract and ovalbumin, and by different routes of exposure to these antigens. Live *Anisakis* induced much stronger systemic Th2 cytokine responses than *Anisakis* extract or OVA administered either orally, intraperitoneally or by epicutaneous application. Subsequently, exposure to live *Anisakis* was the most effective at sensitizing mice to have allergic reactions after the oral ingestion of *Anisakis* proteins. This strong sensitizing effect of live *Anisakis* could be due to factors such as its prolonged presence in the peritoneal cavity, the release of proteolytic enzymes and/or tissue damage caused by the parasite. However, once sensitized by live infection, mice responded to ingestion of *Anisakis* extract alone. Using intraperitoneal or epicutaneous sensitization, *Anisakis* extract behaved similarly to the model allergen OVA, although *Anisakis* extract tended to induce higher levels of total IgE than OVA, suggesting an intrinsic capability of *Anisakis* proteins to induce polyclonal IgE. Another point of interest is that epicutaneous sensitization with *Anisakis* extract consistently induced higher levels of specific IgE than intraperitoneal sensitization or even infection with live *Anisakis* larvae. This highlights the fact that the skin may be an important route of sensitization, particularly in fish processing workers.

In conclusion, the data presented in this thesis have clarified certain aspects of *Anisakis*-induced allergy, and contributed to the knowledge on the distinct roles of IL-4, IL-13 and the IL-4R α in various allergic diseases.

APPENDIX A - Reagents

Anaesthetic

1.2ml ketamine (Anaket-V)

0.8ml xylazine (Rompun)

8ml PBS.

DEPC treated water

1ml diethyl-pyrocabonate

1 litre of water

Caution: Work in the fume cupboard as the fumes from DEPC are toxic. Wear gloves as the skin is full of RNAses, and use RNase-free tips.

Take the DEPC bottle from the fridge and let it warm to room temperature. Carefully remove the cap and stopper.

Add the DEPC to the ddH₂O (or any other solution being DEPC-treated) at a final concentration of 0.1%. Shake well to disperse DEPC. Incubate at 37°C overnight with shaking. Autoclave to sterilize and heat deactivate the DEPC.

Discombe's fluid

0.1% eosin

10% acetone

0.004M EDTA

ELISA Block Buffer

2% milk powder (20g)

0.02% NaN₃ (0.2g)

1xPBS (100ml of 10x PBS)

Make up to 1 litre and store at 4°C.

ELISA Dilution Buffer

1% BSA (10g)

0.02% NaN₃ (0.2g)

1x PBS (100ml of 10x PBS)

Make up to 1 litre and store at 4°C.

ELISA Substrate buffer

0.2g NaN₃

0.8g MgCl₂.6H₂O

Dissolve in 700ml ddH₂O.

Add 97ml di-ethanolamine.
Adjust the pH to 9.8 with 10M HCl.
Make up to 1 litre and store at 4°C.

ELISA wash buffer, 20x

20g KCl
20g KH₂PO₄
144g Na₂HPO₄·2H₂O
800g NaCl
50ml Tween 20
100ml 10% NaN₃

Make up to 5L with ddH₂O.

Formaldehyde denaturing 1% agarose gel

For a 100ml gel:

1g agarose
73.2ml DEPC water
10.0ml MEA electrophoresis buffer
16.8ml 12.3M Formaldehyde (30-40% w/v)

Add agarose to water and melt in the microwave.

Let the melted agarose cool slightly so that it is comfortable to touch the flask (about 60°C).

Add the 10x MEA buffer and mix well.

CAUTION: perform the following steps in a fumehood as the formaldehyde fumes are noxious.

Add the formaldehyde and mix well.

Pour the gel (in the fumehood) and allow to set.

Gel running marker (6x)

50% (v/v) glycerol (diluted in DEPC-treated H₂O)
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
10mM EDTA (pH 8.0)

Iscoe's Modified Dulbecco's Medium (IMDM)

Dissolve 1 tube (17.66g) IMDM in 750ml ddH₂O.
Add 81.7ml of 37g/L NaHCO₃.
Add 2ml of 500x penicillin/streptomycin (P/S).
Using 1N NaOH, adjust pH to between 7.2 and 7.4.
Make up to 1 litre. Filter sterilize and store at 4°C.

Just before use: add 50ml heat-inactivated FCS and 5ml 200mM L-glu per 500ml. Filter-sterilize.

Lethal anaesthetic

500µl Rompun (20mg xylazine/ml stock) Bayer
2ml Anaket V (100mg ketamine/ml stock)
9 ml PBS

MEA electrophoresis buffer

0.2M MOPS (pH 7.0)
20mM sodium acetate
10mM EDTA (pH 8.0)

For 10x solution:

Dissolve 41.8g of MOPS in 700ml of sterile DEPC-treated H₂O. Adjust the pH to 7.0 with 2N NaOH. Add 20ml of DEPC-treated 1M sodium acetate and 20ml of DEPC-treated 0.5M EDTA (pH 8.0). Adjust the volume of the solution to 1 litre with DEPC-treated H₂O. Add DEPC to 1% and incubate overnight at 37°C with shaking. Autoclave to heat deactivate the DEPC and to sterilize. Store at 4°C wrapped in foil to protect from light.

Phosphate-buffered saline (PBS)

80g NaCl
3g KCl
14.4g Na₂HPO₄
2.4g KH₂PO₄

Dissolve the above chemicals in 900ml distilled water.

Adjust to pH 7.4.

Make up to 1 litre.

Filter sterilize.

PNP Substrate

Add 0.5g of 4-nitrophenyl phosphate disodium salt hexahydrate (PNP) to 50ml of ELISA substrate buffer. Mix.

Red Cell Lysis Buffer

8.34g NH₄Cl
0.037g EDTA
1g NaHCO₃

Dissolve in 1 litre of distilled water.
Filter sterilize. Store at 4°C.

RNA loading buffer

Add 7.4µl of loading buffer to 2.6µl of RNA.

Reagents	For 10 reactions
12.3M Formaldehyde	14µl
Formamide	50µl
10x MEA buffer	10µl
10mg/ml ethidium bromide	1µl

Tris-Triton buffer

100mM Tris
0.1% Triton-X
Adjust to pH 8.0.

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APPENDIX B - Neutralization of Cell Samples for TGF- β ELISA

- Prepare 1N HCl and acidify to pH 3.
- Pipette 48 μ l of sample (e.g. cell supernatant) into an Eppendorf tube.
- Add 2 μ l of the prepared 1N HCl to sample (1:25 dilution).
- Incubate the acidified sample at 22°C (RT) for 15 minutes or at 4°C for 60 minutes.
- From these acidified, incubated samples, pipette 48 μ l into an Eppendorf tube and add 2 μ l of 1N NaOH (1:25 dilution).

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APPENDIX C – Glossary

acanthosis – thickening of the epidermal layer; an increase in the thickness of the stratum spinosum (prickle cell layer) of the epidermis

anaphylaxis – a severe, systemic immediate-type hypersensitivity reaction involving multiple organ systems

angioedema – oedema affecting the deeper layers of the skin, or subcutaneous or submucosal tissues

anoxia – lack of oxygen

cetaceans – an order of sea mammals composed of whales, dolphins and porpoises

cornification – the maturation process of epidermal cells (also known as keratinization)

cutis – the whole skin

cytokine – a non-antibody protein released by a cell in response to an antigen which acts as an intercellular mediator

cyanosis – blue tinge to the skin due to lack of oxygen

Der p 1 – a major house dust mite allergen

dermatitis – inflammation of the skin

dermis – the layer of skin between the epidermis and subcutis, composed of connective tissue, blood vessels, lymph vessels, nerve endings and glands

epidermis – the outer layer of skin, a cornifying stratified squamous epithelium

erythema – reddening of the skin (caused by congestion of the capillaries)

excoriation – erosions and ulcers caused by self-trauma

exocytosis – the appearance of migrating inflammatory cells in the epidermis

fibrosis – excessive deposition of fibrous connective tissue

helminth – parasitic worm

hyperaemia – increased blood flow to a part due to dilatation of blood vessels, resulting in reddening and/or engorgement

hyperkeratosis – increased thickness of the stratum corneum

hyperplasia – an abnormal increase of cells in a tissue or organ

hypertrophy – an abnormal increase in cell size

hypovolaemic shock – shock due to loss of blood volume

hypoxia – insufficient oxygen

hypoxaemia – insufficient oxygen in the blood

idiopathic – of unknown cause

interleukin – an immunoregulatory molecule which has effects within the lymphopoietic system

keratinocyte – epidermal cell undergoing maturation

keratosis – a condition of excessive development of horny tissue

lichenification – a thickening of the superficial layers of the skin characterized by exaggerated crevices

macule – a circumscribed change in colour of the skin, less than 1cm diameter

nematode – unsegmented worm with a rounded body and pointed end, belonging to class Nematoda

oedema – excessive intercellular accumulation of fluid in tissues or body cavities

papule – solid elevation of the skin less than 1cm diameter

parakeratosis – abnormal cornification; cells of the stratum corneum (keratinocytes) retain nuclei

plaque – flat topped swelling more than 1cm in diameter

pruritic – itchy

pruritis – intense and persistent itchiness

scale – accumulation of fragments of stratum corneum

spongiosis – intercellular oedema of the epidermis

stratum corneum – outermost layer of the epidermis, composed of dead skin cells (cornified squamous epithelium)

stratum germinativum (also called stratum basale) – germinative layer at the base of the epidermis, composed of living keratinocytes that will gradually grow closer to the surface and cornify (keratinize) to form the stratum corneum. Is composed of simple, columnar epithelial cells on a basement membrane.

stratum granulosum – layer of the epidermis composed of 1-3 rows of flattened, granular cells, the last living layer of cells

stratum lucidum – a layer of the epidermis composed of a single row of freshly made cornified squamous epithelial cells

stratum spinosum – layer of the epidermis beneath the stratum granulosum, composed of multiple layers of cuboidal cells called prickle cells. Also known as the prickle cell layer.

urticaria – wheals induced by histamine release, hives

vasodilation – dilation of the blood vessels

wheal – a sharply demarcated raised lesion caused by dermal oedema

zoonosis – a disease of animals that may be communicated to man

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