Investigating the immunological response elicited to the gastrointestinal nematode pinworm (*Syphacia obvelata*)

Chesney Elroy Michels

2006

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University of Cape Town

Investigating the immunological response elicited to the gastrointestinal nematode pinworm (*Syphacia obvelata*)

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A project proposal in partial fulfilment of the requirement for the degree of PhD

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Table of Contents

Acknowledgements iii
Abbreviations iv
List of tables and figures vii

Abstract x

Thesis Objectives xi

Chapter I – *Pinworm – Syphacia obvelata* 1

Chapter II – *Pinworm influences Ova-induced anaphylaxis* 104

Chapter III – *Diagnostic analysis* 136

Conclusion 182

Future research 183

Appendix A 184
Appendix B 195

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Abbreviations

α	Alpha
β	Beta
δ	Delta
γ	Gamma
η	Eta
μ	Micro
ζ	Sigma
γ_c	common γ chain
~	approximately
%	percentage
aa	amino acids
Ab	Antibody
ADCC	Antibody-Dependent Cytotoxic
Ag	Antigen
AP	Alkaline Phosphatase
APC	Antigen Presenting Cell
BCR	B cell antigen receptor complex
BSA	Bovine Serum Albumin
CD	Cell-associated Differentiation
CFA	Complete Freund's Adjuvant
cm	centimeter
CRD	Carbohydrate-Recognition Domain
CRP	C-reactive Protein
CTL	Cytotoxic T Lymphocytes
^{0}C	degrees Celsius
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DR	Dioxin Receptor
ECP	Eosinophil Cationic Protein
EDTA	Ethylene Diamine Tetra-Acetic acid
ELISA	Enzyme-linked Immunosorbent Assay
EPO	Eosinophil Peroxidase
FCS	Fetal Calf Serum
FBG	Fibrinogen β-gamma
FcγR	IgG Fc-domains
FcεRI	high affinity IgE receptor
F/T	Freeze and Thaw

G	Gauge
G-CSF	Granulocyte Colony-Stimulating Factor
GI	Gastrointestinal
GIT	Gastrointestinal tract
GALT	Gut-Associated Lymphoid Tissue
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HE	Haematoxylin and Eosin
hr	hour
HRP	Horseradish peroxidase
IFA	Incomplete Freund's Adjuvant
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IMDM	Iscove's Modified Dulbecco's Medium
i.p.	intraperitoneal
IL	Interleukin
IRS	Insulin Receptor Substrate
kDa	kilodalton
kg	kilogram
KO	Knock-out
L	Liter
LDL	Low-Density Lipoprotein
LFA	Lymphocyte Function-associated Antigen
LPS	Lipopolysaccharide
LT	Leukotriene
M	Molar
MASP	(MBL)-Associated Serine Protease
MBL	Mannose-Binding Lectin
MBP	Major Basic Protein
M-CSF	Macrophage-Colony-Stimulating Factor
mg	milligram
MHC	Major Histocompatibility Complex
min	minute
MIP	Macrophage Inflammatory Protein
MLN	mesenteric lymph node
μl	microliter
ml	milliliter
mM	millimolar
NK	Natural Killer
OD	Optical Density

O/N	Overnight
PAF	Platelet Activating Factor
PAMP	Pathogen-Associated Molecular Patterns
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cells
PEG	Polyethylene glycol
PG	Prostaglandin
pI	Isoelectic point
Pin	Pinworm
Pin/Ova	Pinworm/Ovalbumin
PRR	Pattern Recognition Receptor
RAG	Recombinase-Activating Gene
RCLB	Red Cell Lying Buffer
RELM	Resistin-Like Molecule
rpm	revolution per minute
RT	Room Temperature
rxn	reaction
SAP	Serum Amyloid Protein
s.c.	subcutaneous
SCID	Severe Combined Immuno-Deficient mice
SDS	Sodium Dodecyl Sulfate
sec	second
SLE	Systemic Lupus Erythematosus
SOPS	*Syphacia obvelata* Polyclonal Serum
spp.	species
SR	Scavenger Receptors
Stat-4	Signal transducer and activator of transcription 4
Stat-6	Signal transducer and activator of transcription 6
TCR	T Cell Receptor
TDTH	Delayed-Type Hypersensitivity T cells
TE	Tris/EDTA
TGF	Transforming Growth Factor
Th1	T helper 1
Th2	T helper 2
TLP	Toll-Like Receptor
TNF	Tumor Necrosis Factor-alpha and lymphotoxin
U	unit
VLA	Very Late Antigen
wt	wild type

List of tables

Page

Table 1.1: Eosinophilia, mastocytosis and goblet cell hyperplasia in responds to *Syphacia obvelata* infection. 62

List of figures

Figure 1.1: Model receptors of the immunoglobulin (Ig) superfamily. 12

Figure 1.2: Simplified diagram representing the sequence of events induced by a humoral and cell-mediated immune response. 12

Figure 1.3: T cell dichotomy in response to an infection. 17

Figure 1.4: IL-4 and IL13 receptor complexes. 34

Figure 1.5: Proposed GI nematodes expulsion model. 39

Figure 1.6: Pinworm lifecycle (*Enterobius vermicularis*). 41

Figure 1.7. Naturally infected mice maintained in a conventional animal facility. 56

Figure 1.8. Antibody isotype profile. 57

Figure 1.9. Worm burden of an experimental infectious model. 59

Figure 1.10. IL-4 and IL-13-mediated functions induce protective immunity to *S. obvelata*. 59

Figure 1.11. Immune response to helminth in BALB/c and IL-4R$\alpha^{-/-}$ mice. 61

Figure 1.12. *S. obvelata*-specific IgG1 and IgG2b levels. 62

Figure 1.13. *S. obvelata* worm burden in cell-specific gene-targeted mice. 64

Figure 2.1: Simplified schematic of an anaphylactic reaction. 110

Figure 2.2: Classification of hypersensitivity reactions, type I-IV. 112

Figure 2.3: Mechanisms of systemic anaphylaxis. 115

Figure 2.4: *S. obvelata* influences Ova-induced anaphylaxis. 121

Figure 2.5: Ova-specific IgE and IgG1 antibody response. 122

Figure 2.6: Degranulation of mouse mast cell protease-1. 122

Figure 2.7: *S. obvelata* reduce Ova-specific IL-4 and IL-5 cytokine production. 124

Figure 2.8: *Syphacia obvelata* antigen is non-antigen specific inducing both a cytotoxic and inhibitory effect on cells. 125

Figure 3.1: Schematic diagram representing the universally used indirect and direct ELISAs for antibody and Ag detection. 143

Figure 3.2: The Y-shaped monomer of an immunoglobulin molecule which consists of two identical heavy chains and two identical light chains. 144

Figure 3.3: Schematic diagram of the stages involved in the association of an antibody with an antigen. 145

Figure 3.4: Stages of monoclonal Ab production. 148

Figure 3.5: An end-point titration to determine a standard *S. obvelata* coating concentration. 164

Figure 3.6: Anti-*S. obvelata* hyper-immune serum was generated in BALB/c mice for use as positive control and arbitrary standard. 165

Figure 3.7: Cross-reactivity of anti-*S. obvelata* serum against Ag of other research parasites. 167

Figure 3.8: Rabbit anti-SOPS antibody is an effective diagnostic tool for detecting pinworm. 169

Figure 3.9: Production and analysis of monoclonal rat anti-*S. obvelata* antibody. 171

Figure 3.10: Antigenic protein profile of the Ags derived from *S. obvelata* in response to immunoglobulin isotypes. 172

Abstract

It is important to emphasize with the advance of biotechnology and increased global exchange of animals and animal products, the risks of introducing adventitious infections. Previous studies of specific-pathogen-free mouse colonies have identified the presence of infectious agents in 10-35% of research institutions investigated. Prevalence was higher among non-SPF mice with pinworm reported in 70% of institutions housing rodents under these conditions. Pinworm, a gastrointestinal (GI) nematode is commonly found in laboratory animals. The direct transmission of the parasite by contaminated food, water and bedding result in their continual re-exposure to the host, making the control of pinworm in animal holdings quite difficult. *Syphacia obvelata*, mouse pinworm, has been shown to interfere with research goals in several experimental models. In this study, we show the consequence of a pinworm outbreak in a transgenic barrier facility and define the immune response elicited in BALB/c mice. Infection with *S. obvelata* induced a transient Th2-type immune response with elevated cytokine production and parasite-specific IgG1. In contrast, BALB/c mice, deficient for IL-13, IL-4/13 or IL-4Rα showed chronic disease with more than 100-fold higher parasite burden, increased IFN-γ production, parasite-specific IgG2b and a default Th2 response. Notably, infected IL-4$^{-/-}$ BALB/c mice showed only slight elevated parasite burden compared to controls, suggesting that IL-13 plays the dominant role in the control of *S. obvelata*. Furthermore, no significant eosinophilia, mastocytosis or goblet cell hyperplasia was induced.

In a well-established ovalbumin (Ova) anaphylaxis model, we show that mice infected with *S. obvelata* induce a more severe anaphylactic reaction, with consistently greater temperature decline than their non-infected counterparts. Analysis of spleen cells further revealed a marked reduction of Ova-specific Th2 cytokines, highlighting the importance of pinworm free experimental mice.

Finally, we generated anti-*S. obvelata* antibody to optimize the detection ELISA and identified target epitopes for future analysis.

In conclusion, we identify the T helper immune response induced to *S. obvelata* and demonstrate the importance of IL-13 for the expulsion of the GI nematode. We show that *S. obvelata* induces a non-protective immune response to a common food allergen and confirm that the pinworm-specific ELISA is an effective diagnostic tool for detecting pinworm infected mice.

Thesis Objectives

1. After observing some irregularities in experiments from mice housed in one of our conventional animal facilities, we isolated a gastrointestinal nematode, *Syphacia* sp. In this study, we aimed to optimize the isolation of the GI nematode, identify the species and determine the immune response induced in mice infected.

2. Determine whether infection with the *Syphacia* species influence non-related experimental disease models, using a well established food allergy model (ovalbumin).

3. Establish an optimal detection system to identify the *Syphacia* species early in mice and prevent or limit an outbreak.

Chapter I

Syphacia obvelata induces a protective Th2 immune response

Table of contents

1.1 Summary 5

1.2 Introduction 7

1.2.1 The immune system 7
1.2.1.1 Innate immunity 7
1.2.1.2 Adaptive immunity 8
1.2.1.3 Antigen recognition 9
1.2.1.3.1 B-cell receptor 10
1.2.1.3.2 T-cell receptor 10
1.2.1.3.2.1 αβT cells 10
1.2.1.3.2.2 γδT cells 11
1.2.1.4 Humoral immune response 11
1.2.1.5 Cell-mediated immune response 13
1.2.1.6 Cytokines 18
1.2.1.6.1 Type I receptor family 19
1.2.1.6.2 Type II receptor family 19
1.2.1.6.3 Type III receptor family 19
1.2.1.6.4 Type IV receptor family 20
1.2.1.6.5 Chemokine receptor 20
1.2.2 Parasites 21
1.2.2.1 Parasite classification 21
1.2.2.1.1 Phylum Sarcomastigophora 21
1.2.2.1.1.1 Mastigophora 21
1.2.2.1.1.2 Sarcodina 22
1.2.2.1.2 Phylum Ciliophora 22
1.2.2.1.3 Phylum Apicomplexa 22
1.2.2.1.4 Phylum Microspora 23
1.2.2.1.5 Phylum Platyhelminthes 23
1.2.2.1.5.1 Trematoda 23

1.2.2.1.5.2 Cestoda 24
1.2.2.1.6 Phylum Arthropoda 24
1.2.2.1.6.1 Arachnida 24
1.2.2.1.6.1.1 Ticks 24
1.2.2.1.6.1.2 Mites 25
1.2.2.1.6.2 Insecta 25
1.2.2.1.6.2.1 Mosquitoes 25
1.2.2.1.6.2.2 Sandfly 25
1.2.2.1.6.2.3 Tsetse fly 25
1.2.2.1.7 Phylum Aschelminthes 26
1.2.2.1.7.1 Nematoda 26
1.2.2.2 Parasite immunology 29
1.2.2.2.1 Immunity to gastrointestinal (GI) nematodes 30
1.2.2.2.1.1 Innate immunity to GI nematodes 30
1.2.2.2.1.1.1 Gastrointestinal mucins 31
1.2.2.2.1.1.2 Pattern recognition molecules 31
1.2.2.2.1.1.2.1 Collectins and ficolins 31
1.2.2.2.1.1.2.2 Pentraxins 32
1.2.2.2.1.1.2.3 C-type lectins 32
1.2.2.2.1.1.2.4 Scavenger receptors 32
1.2.2.2.1.1.2.5 Complement receptors 32
1.2.2.2.1.1.2.6 Toll-like receptor 33
1.2.2.2.1.2 Adaptive immunity to GI nematodes 33
1.2.2.2.1.2.1 Protective Th2 immune response 33
1.2.2.2.1.3 GI nematode expulsion 35
1.2.2.2.1.3.1 Eosinophils 36
1.2.2.2.1.3.2 Mast cells 36
1.2.2.2.1.3.3 Smooth muscle cells 36
1.2.2.2.1.3.4 Goblet cells 37
1.2.2.2.1.3.5 Intestinal epithelial cell turnover 37
1.2.2.2.1.3.6 Antibody 38

1.2.3 Pinworm 40
1.2.3.1 *Enterobius vermicularis* 40
1.2.3.1.1 *Enterobius vermicularis* as a probable vector for *Dientamoeba fragilis* 42
1.2.3.2 *Syphacia* species (rodent pinworm) 43
1.2.3.2.1 Influence of pinworm on experimental disease models 43
1.2.4 Hypothesis 45

1.3 Material and Methods 46

1.3.1 Mice 46
1.3.2 Culturing of *S. obvelata* 46
1.3.2.1 Stahl's method 46
1.3.3 Egg and antigen preparation 46
1.3.3.1 Eggs 46
1.3.3.2 Antigen 47
1.3.4 Infection 47
1.3.5 Antibody response 47
1.3.5.1 Collecting blood 47
1.3.5.1.1 Anesthesia with exsanguinations 47
1.3.5.1.2 Tail vein bleed 48
1.3.5.2 Antibody ELISA 48
1.3.6 Protein concentration (BCA kit) 49
1.3.7 Isolation of organs 49
1.3.8 Cell restimulation 49
1.3.9 Analysis of cytokine ELISAs 50
1.3.10 Eosinophilia 51
1.3.10.1 Peripheral blood eosinophilia 51
1.3.10.2 Eosinophil peroxidase (EPO) assay 51
1.3.10.2.1 Tissue isolation 51
1.3.11 Intestinal mastocytosis 52
1.3.11.1 Mouse mast cell protease-1 assay 52

1.3.12 Periodic Acid Schiff (PAS) staining technique for carbohydrate 52
1.3.12.1 Preparation of samples 52
1.3.12.2 Staining 52
1.3.13 Statistics 53

1.4 Results 54

1.4.1 An outbreak of pinworm identified type 2 immune responses protective 54

1.4.2 Isotype specific antibody response to mice naturally infected with *S. obvelata* 55

1.4.3 *Syphacia obvelata* resistance is IL-4Rα-dependent 58

1.4.4 Endogenous IL-13 and not IL-4 is dominant cytokine eliciting protection 58

1.4.5 *S. obvelata* induces a Th-2 immune response 60

1.4.6 Eosinophilia, mastocytosis and goblet hyperplasia 63

1.4.7 IL-13 signaling via IL-4Rα on macrophages, T cells and smooth muscle cells are not required for protection against *S. obvelata* 64

1.5 Discussion 65

1.6 Reference 72

1.1 Summary

Vertebrates have an extensive immune system that has evolved to defend the host against a spectrum of diseases, many of which arise from infectious agents such as bacteria, viruses and parasites. Parasites include protozoa and helminths, the latter of which infect billions of people worldwide but are of particular concern in developing countries. Gastrointestinal (GI) nematodes, one of the most commonly acquired helminth infections, are prevalent in communities where poverty and low sanitation is present. Individuals living in endemic areas and under these condition often harbor more than one species of parasite throughout their lives. In these communities, children are the most vulnerable as recurring infections at an early age have been associated with stunted growth, slow mental development and malnutrition, and should therefore be the target of control programs.

In this study, we addressed the immune response to a rapidly spreading intestinal nematode, pinworm. The human species, *Enterobius vermicularis*, is widely recognized as a common parasite of the intestine, with disease causing severe pruritus (intense itching sensation) that may lead to secondary bacterial infections. *Syphacia obvelata*, the mouse species investigated in our model is a frequent contaminant of both specific pathogen free and conventional animal facilities. Although the immune response induced by this GI nematode had not been described before, concurrent infections with the worm have been shown to interfere with research goals in several experimental models.

In this study, we show the consequence of a pinworm outbreak in a transgenic barrier facility and define immune response induced by the infection in BALB/c mice. Infection with *S. obvelata* induced a transient Th2-type immune response with elevated IL-4, IL-5, IL-9, IL-10 and IL-13 cytokine production and parasite-specific IgG1. In contrast, BALB/c mice, deficient for IL-13, IL-4/13 or IL-4Rα showed chronic disease with more than 100-fold higher parasite burden, increased IFN-γ production, parasite-specific IgG2b and a default Th2 response. Interestingly, infected IL-4$^{-/-}$ BALB/c mice showed only slightly elevated parasite burden compared to

controls, suggesting that IL-13 plays the dominant role in the control of *S. obvelata*. No significant eosinophilia, mastocytosis or goblet cell hyperplasia was induced suggesting that none of these effector mechanisms dominant at expelling the nematode. In conclusion, we describe for the first time, the T helper immune response to *S. obvelata* and demonstrate the importance of IL-13 for the expulsion of this GI helminth.

1.2 Introduction

1.2.1 The immune system

The immune system is an advanced network that protects vertebrates from invading pathogens and other foreign molecules [1,2]. In response to an infection, a cascade of specific and complex pathways are induced, the collective purpose of which is to neutralize and ultimately eliminate foreign material, while strictly discriminating and tolerating self-structures. Each response is a unique sequence of events, shaped by the nature of the challenge [2]. The immune system consists of two lines of defense namely; innate immunity and adaptive immunity.

1.2.1.1 Innate immunity

The innate immune system can be subdivided into the following:

(a) Biological barriers (inflammation, phagocytosis),

(b) Chemical barriers (enzymatic action, interferons, beta-lysin, fibronectin, complement),

(c) General barriers (fever), and

(d) Physical barriers (skin, mucous membrane)

Entry of infectious material into the host induces the production of proinflammatory cytokines (IFN-γ, IL-12, TNF-α) that activate macrophages and dendritic cells ('professional' phagocytic cells). These cells identify the invaders and co-operate to destroy foreign material. As antigen presenting cells (APC) they concentrate foreign material into specialized lymphoid tissues (lymph nodes and spleen) for recognition by T and B cells [2].

1.2.1.2 Adaptive immunity

An adaptive or specific immune response is much slower than and dependent on innate immune response for antigen presentation of potential pathogens. Importantly, it has the ability to remember previously encountered infectious material and respond more efficiently upon further exposure. The pivotal players of this immune response are lymphocytes.

Lymphocytes are small, non-phagocytic cells that lack stainable cytoplasmic granules and are found in lymphatic tissues (lymph nodes, spleen, liver) [2]. They are characterized by their restricted range of receptors, which allow each cell to respond to an individual antigen (specificity), and respond through clonal proliferation of long living memory cells. These cells undergo continuous recirculation from tissues into the bloodstream, enabling a rapid memory response, following re-exposure to the antigen distributed throughout the body [3].

Undifferentiated lymphocytes are derived from bone marrow progenitor cells (hemopoietic stem cells) [2] and mature into the three major subpopulations of an adaptive immune response: the T, B and null cells.

T lymphocytes (T cells) migrate in their undifferentiated form to the thymus where a selected population of 5% matures. They undergo special processing followed by a selection to become one of the four principle T cell types. These are: **(a)** Inducer T cells which oversee the development of T cells in the thymus, **(b)** helper T cells (Th), that initiate any response, **(c)** cytotoxic T cells, to eliminate host cells that have been infected, and **(d)** suppressor T cells, which terminate an immune response [4].

B lymphocytes (B cells) differentiate in the fetal liver and adult bone marrow and are distributed from these tissues by blood and lymph. In peripheral lymphoid tissues, B cell populations are heterogeneous, distinguished by the different cell surface molecules they express. Part of their heterogeneity results from B-cell maturation in response to antigenic stimulation [5]. Upon encountering an antigen, B cells are

detained in T cell areas and may be activated to proliferate by appropriate Th cells. Some of these activated B cells differentiate into plasma cells while others migrate to a nearby lymphoid follicle where they establish germinal centers. Here, the activated B cells undergo intense proliferation and optimization of receptor binding to produce cells with a higher affinity for the stimulating antigen [5].

Null cells are the third subpopulation of cells, and resemble lymphocytes but lack the characteristics of T and B cell markers. These cells are distinguished by the presence of cytoplasmic granules, and it is currently believed that they consist of early T and B cells, monocytes, natural killer cells (NK) and antibody-dependent cytotoxic T-cells (ADCC) [2,3]. Although very little is known about the functions of these cells, they are thought to be involved in tumor, virus and parasite immunity [3].

1.2.1.3 Antigen recognition

Antigen are molecules specifically recognized by the adaptive elements of the immune system that is B cells, T cells or both [6]. Antibodies which recognize and react with antigens are specialized glycoproteins produced either as a B cell receptor (Fig. 1.1) or as secreted molecules [1]. T cell receptors (TCR) exists only as an integral membrane protein [1]. The specialized glycoproteins of B cells have common structural features that are produced by gene recombination, a complex regulated gene translocation mechanism that results in one antigen binding specificity on each cell (i.e. each secreted antibody or B cell recognizes and respond to a unique antigen). This mechanism results in a repertoire of diverse antibody/receptor that can distinguish over 10^7 antigenic epitopes (restricted part of the antigen). For that reason, there might be several different antibodies for a given pathogen, each binding to a different antigen on that pathogen's surface. Furthermore, a particular antigen could have several different epitopes or repeated epitopes, to which antibodies are specific.

1.2.1.3.1 B-cell receptors

B cells are defined by the presence of surface immunoglobulins (Ig). These molecules are constitutively produced and consist of an extracellular region, which reacts with the antigen, an anchor which binds the receptor in the cell membrane, and a cytoplasmic part, through which the receptor transmits signals into the cell [7]. The majority of human B cells in the peripheral blood express IgM and IgD isotype antibody on their surface. The antigen binding sites of receptor (IgM and IgD isotype) are identical and have the same binding specificity as the antibody produced by the cell [8]. When Ig associates with other molecules on the B cell surface, they form the 'B-cell antigen receptor complex' (BCR). These 'accessory' molecules consist of disulphide-bonded heterodimers of Igα (CD79a) and Igβ (CD79b). They interact with the transmembrane segments of the Ig receptor and are involved with cellular activation (Lydyard, 2001 #16).

1.2.1.3.2 T-cell receptors

Two distinct types of T-cell antigen receptors exist; one, a heterodimer of 2 disulphide-linked polypeptides (α and β); the other, a structurally similar molecule but composed of γ and δ polypeptides [8]. Both receptors are associated with a set of 5 polypeptides, the CD3 complex, and together form the T-cell receptor (TCR:CD3 complex; Fig. 1.1).

1.2.1.3.2.1 αβT cells

αβT cells account for 90-95% of blood circulating T cells and are further subdivided into 2 distinct non-overlapping populations. A subset defined by the CD4 marker and mainly 'helps' or 'induces' immune responses and a subset carrying the CD8 marker and is predominately cytotoxic. $CD4^{+}$T cells are restricted to specifically recognize their antigen in association with major histocompatibility complex (MHC) class II molecules, whereas $CD8^{+}$T cells recognize antigen associated with MHC class I molecules [8].

1.2.1.3.2.2 γδT cells

The majority of intraepithelial lymphocytes (IELs) are γδT cells expressing CD8, a marker not usually found on circulating γδT cells. However, these cells ($CD8^+$ γδT cells) have a specific repertoire of T-cell receptors biased towards certain bacterial/viral antigens and are believed to play an important role in protecting the mucosal surfaces of the body. Some γδT cells may recognize antigens directly, without the need for APC [8].

1.2.1.4 Humoral immune response

B cells secrete antibodies generally directed towards extracellular antigens (Fig. 1.2) [2]. Once a B cell receptor recognizes and binds an antigen to which it is targeted, it begins to divide rapidly and its progeny differentiate into plasma and memory cells. Mature plasma cells have a well developed rough endoplasmic reticulum characteristic of cells producing protein for 'export' [9]. They are miniature antibody factories potentially producing 2000 antibodies per second and have a life span of 5 days (Fig.1.2). Memory B cells having an increased affinity for antigen ensure that re-infection is dealt with swiftly [10].

Antibodies are not capable of destroying pathogens in their own right, instead they act as activators and inducers of pathogen eliminating pathways, which include complement activation, opsonization and immune complex formation [2].

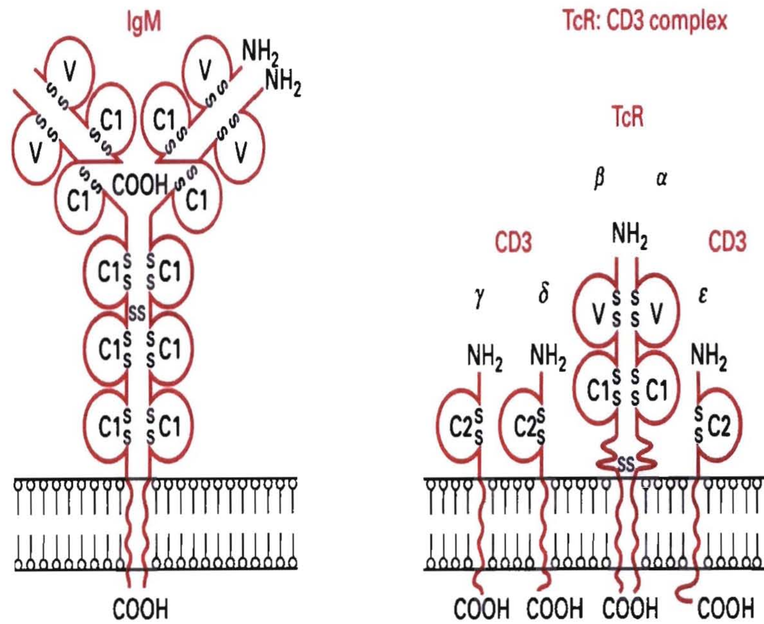


Figure 1.1: Model receptors of the immunoglobulin (Ig) superfamily. The circles represent Ig domains. Membrane IgM represents a structural monomer of both a secretary and membrane Ig molecule. TCR-CD3 is the T-cell receptor with the co-receptor CD3. This figure has been adapted from Williams [11].

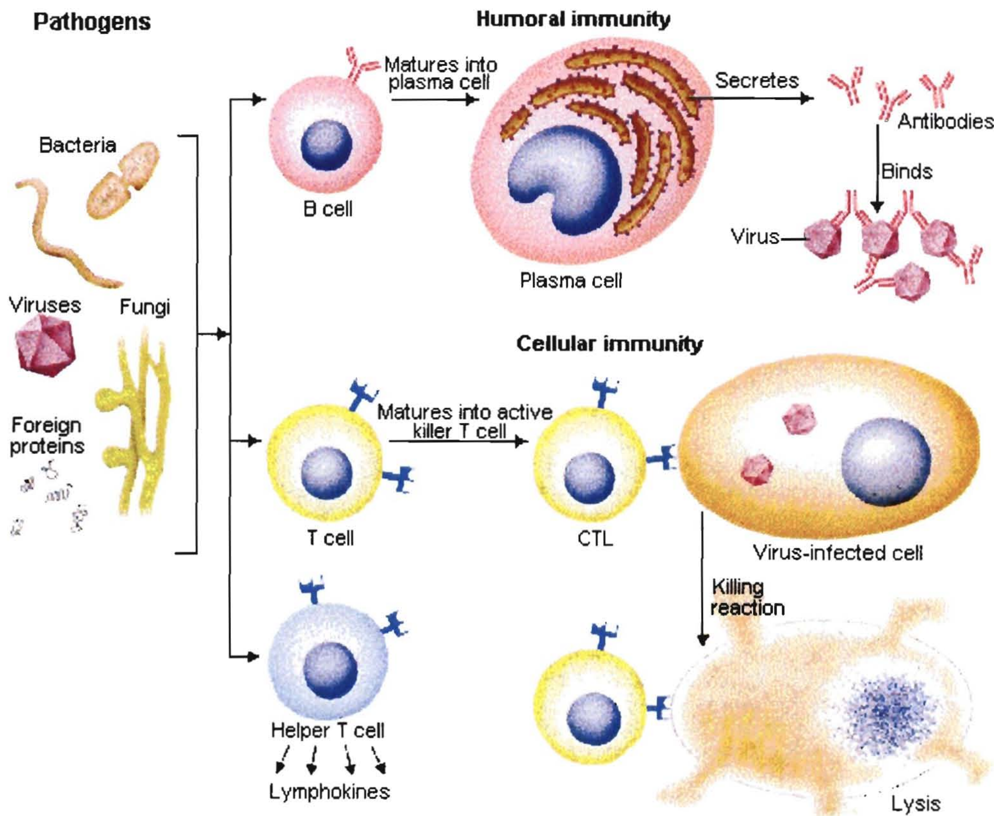


Figure 1.2: Simplified diagram representing the sequence of events induced by a humoral and cell-mediated immune response. This figure was taken directly from www.tmd.ac.jp [12].

1.2.1.5 Cell-mediated immune response

This type of immune response requires cell-cell interaction and is involved in immunity towards intracellular pathogens, graft rejection, chronic inflammation and tumors. T cells controlling these responses have specialized multi-chain receptor complexes (γ, ε, η, δ, ζ chains) and cell surface markers (CD2, VLA, LFA-1, CD28, CD4 and/or CD8) that recognize and bind antigens expressed by MHC molecules on the surface of infected host cells. In order to activate and allow clonal expansion of effector T cells, regulatory molecules known as cytokines have been shown to direct and drive the selection of effector mechanisms. For example, interleukin-2 (IL-2, a T cell growth factor), IFN-γ and TNF (TNF-α and lymphotoxin), which are associated with immunity against intracellular parasites have been shown to activate macrophages, cytotoxic T cells (CTLs) and NK cells, with IL-3, IL-4, IL-5, and IL-9 important in the activation of mast cells, eosinophils and B cells associated with helminth parasites [13,14].

These divergent pathways in the effector mechanism are consistent with the production of different cytokines by $CD4^+$ T cell subsets (T helper cells, Th). $CD4^+$ T cells can be sub-divided into Th1 and Th2 cells (Fig. 1.3) according to the cytokines they produce [15]. Th1 cells produce IL-2, IFN-γ and TNF-α and aid in the production of IgG2a and IgG2b from B cells. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 while driving the production of IgG1 and IgE antibodies [16-19].

A second population of effector T cells, distinguished by their $CD8^+$ receptors is the Cytotoxic T Lymphocytes (CTL) [2]. CTLs have evolved strategic mechanisms to eliminate malignant host cells. Acting synergistically with macrophages and NK cells, the CTLs secrete enzymes (perforin and granzymes) that puncture holes in infected cells disrupting their osmotic balance causing cell lysis (Fig. 1.2). In addition, they produce Fas ligands, enhanced by the presence of TNF-α, and directed towards triggering apoptosis of malignant cells (programmed cell death of infected cells).

Natural Killer cells account for up to 15% of the blood lymphocytes and express neither T-cell nor B-cell antigen receptors [8]. They react against cells, which do not express MHC class I molecules, by recognizing alternative receptors. Upon activation, NK cells release IFN-γ and other cytokines (e.g. IL-1 and GM-CSF), which may be important in the regulation of haemopoiesis and immune responses [8].

Macrophages are phagocytic cells participating both in specific immunity via antigen presentation and IL-1 production, and non-specific immunity against bacterial, viral, fungal, and neoplastic pathogens [20]. They are derived from bone marrow stem cells and pass into the blood as monocytes [6], before migrating into tissue, and transforming into macrophages [21]. Blood monocytes despite being considered as immature macrophages possess migratory, chemotactic, pinocytic and phagocytic activities, as well as receptors for IgG Fc-domains (FcγR) and iC3b complement. After differentiating into mature macrophages, they participate non-specifically by internalizing pathogens subjecting them to toxic molecules and enzymes within the macrophage phagolysosome (phagosome fused lysosome). Pathogens that are to large to be internalized trigger the release of mediators from macrophages, which induce damage to pathogens within the extracellular environment [6]. Consequently, this response also produces necrosis and inflammation. In addition, activated macrophages secrete TNF which can also induce apoptosis. Thus, macrophages can induce necrosis, apoptosis or a combination of both depending on the state of activation and the target involved.

More recently factors modulating the plethora of biological functions exhibited by macrophages have been investigated. The heterogeneity and state of activation of macrophages are well known to be influenced by microbial antigens together with effector T cells, and their secretory products [22]. The majority of our knowledge stems from classically activated macrophages, which are induced by proinflammatory molecules such as lipopolysaccharide (LPS) in a Th1 cytokine environment (IFN-γ and TNF) and release inflammatory and/or microbicidal products [22]. These cells are characterized by their high antigen-presenting efficiency, ability to produce nitric oxide (NO), a protective response to destroy intracellular pathogens, combined with their increased expression of major histocompatibility complex (MHC) class II and

CD86. Classical macrophages also exert anti-proliferative and cytotoxic activities, with pathological consequences upon host tissue, as these activities result partly from their ability to secrete NO and pro-inflammatory cytokines (TNF, IL-1, IL-6; (Noel, 2004 #264). As a result, the development of an anti-inflammatory mechanism to attenuate excessive inflammation is critical to the immune system, to control infection yet limit tissue damage.

Macrophages arising in a Th2 environment are termed alterative macrophages, and express the macrophage mannose receptor, Arginase I, FIZZ-1 and YM-1 [23-25]. In contrast, to classically activated macrophages, alternative activated macrophages are less efficient at killing intracellular pathogens in mice, as they fail to generate NO from L-arginine [26]. Alternative macrophages characteristically enhanced the expression of particular pattern recognition proteins. In humans, these macrophages specially express the anti-inflammatory IL-1 antagonist (IL-1Rα), IL-1 decoy receptor, alternative macrophage activation-associated CC chemokine-1 (AMAC-1) [27], macrophage-derived chemokine (MDC) [28], and thymus and activation-regulated chemokine (TARC) [29]. These receptors are believed to recruit Th2 cells through their interaction with CC chemokine receptor 4 (CCR4). In the murine model, the genes FIZZ-1 and YM-1 are abundantly expressed during parasite infection. The expression levels of costimulatory molecules are similar for both activated macrophages (CD11a, CD40, CD54, CD58, CD80 and CD86) [30]. However, alternative activated macrophages do exhibit enhanced endocytic and phagocytic ability, increased expression of MHC class II molecules and can perform antigen presentation [31]. Alternative macrophages are antagonistically regulated by Th1 cytokines and although it has not yet been demonstrated, alternatively activated macrophages have been speculated to downregulate inflammatory processes initiated by classically activated macrophages on the basis of secreting anti-inflammatory molecules such as IL-10 and transforming growth factor β (TGF-β) [22]. IL-10 is known to have broad suppressive effects on macrophage cytokine production and costimulatory molecule expression [23,27,32]. It is important to note that the downregulation of the inflammatory immune response might not be a general mechanism but might only hold true for Th1 cytokine driven inflammation. This is owing to increasing evidence indicating that alternatively activated macrophages can induce inflammation which leads to

pathology, as observed in Th2 cytokine controlled inflammatory diseases (e.g. allergies, gastric pathology, parasite infections). Evidently, alternatively activated macrophages have the ability to induce extracellular matrix synthesis which could play a role in wall thickening and is associated with pathological changes during allergic pulmonary inflammation [33].

The role of alternatively activated macrophages seems poised at downregulating the inflammatory immune response with a minimal role if any at expelling nematodes. IL-4/IL13-activated macrophages were shown not to be important for *Nippostrongylus brasiliensis* expulsion [34]. This finding was corroborated by bone marrow reconstitution studies which demonstrated that hematopoietic cells do not mediate worm expulsion [35]. Moreover, alternatively activated macrophages do not play an important role in Th2 cell development as Th2/type 2 responses were normal in *N. brasiliensis* and *Schistosoma mansnoi* infected mice lacking the IL-4/IL13-activated macrophages ($LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice). The unwavering of a protective Th2 immune response particularly IL-13 cytokine production, would explain the expulsion of the GI nematode, *N. brasiliensis*. IL-13-dependent goblet cell hyperplasia expels the worm [36].

Other roles within helminth infections associated with alternatively activated macrophages include their recruitment in high numbers, which suggests that they are important effector cells, releasing molecules that target extracellular helminths and further promote the Th2 immune response [37]. Their repair of damaged tissue caused by large extracellular helminths is also an important function [37].

Another non-lymphoid cytotoxic effector cell is the *eosinophil*. Mature eosinophils characterized by their granules are only weakly phagocytic, with less efficient intracellular killing. Instead, they are effective extracellular killing machines secreting various toxic granule constituents, following activation [38]. The key components of the eosinophil granule protein include major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP). Their degranulation can be triggered in a number of ways, from the binding of IgG-coated parasites via surface $Fc\gamma RII$, to being triggering via $Fc\varepsilon RII$ binding and finally

directly in vitro by several cytokines (IL-3, IL-5, GM-CSF, TNF, interferon-β and PAF) [38].

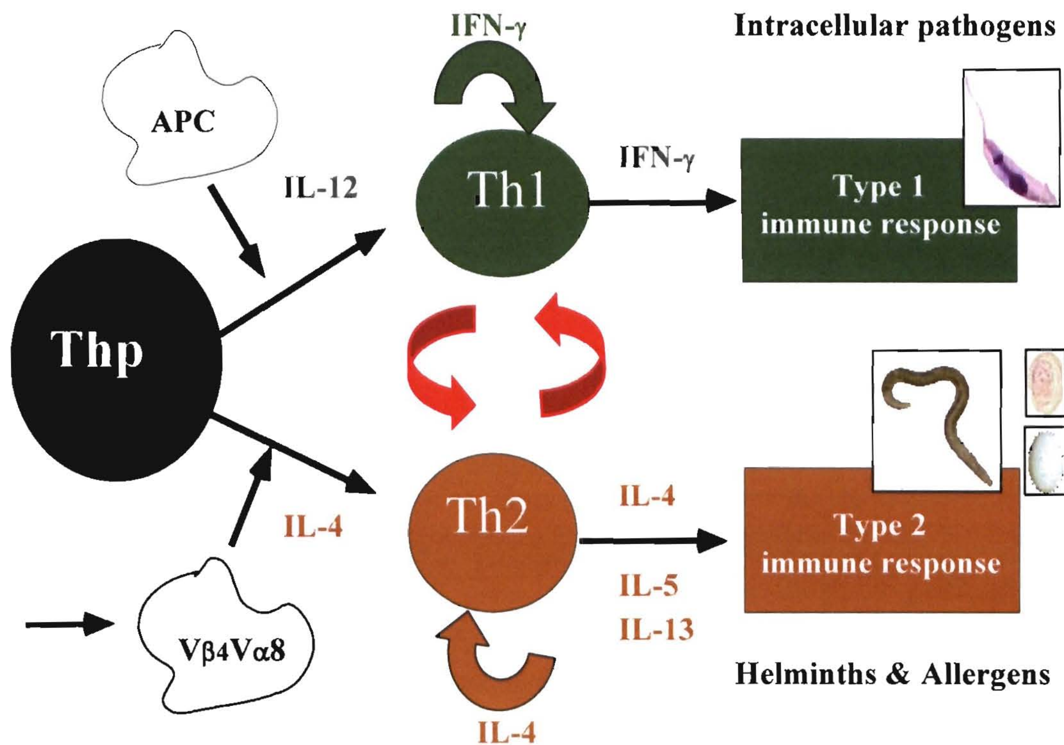


Figure 1.3: T cell dichotomy in response to an infection, adapted from Mohrs et al [39]. IL-12 and IL-4 induced Th1 and Th2 development and their cytokine immune responses respectively. Thp, undifferentiated naïve T cell; Th1, T helper 1 cell; Th2, T helper 2 cell; APC, antigen presenting cell; Vβ4Vα8, IL-4 producing T cell; IL, interleukin; Black, Green and Orange arrows indicate positive effects; Red arrows indicated negative effects

1.2.1.6 Cytokines

The activation of the immune system in response to invading pathogens, foreign antigen or tissue damage, is often accompanied by distinct alterations in neurological and endocrine functions [40]. These systemic responses to injury and infection induce protective immunity orchestrating effector immune cells, including T and B lymphocytes, NK cells, macrophages, dendritic cells and granulocytes [1]. The responses of these effector cells are regulated by small glycosylated soluble polypeptides, called cytokines. Cytokines are intra-and-intercellular (autocrine and paracrine) communication molecules that are composed of members of the interleukin, interferon, TNF, growth factor (e.g. TGF-β), and hematopoietic colony-stimulating factors (e.g. GM-CSF, G-CSF, and M-CSF) [41]. To exert greater control on biological activities, cytokines may act on cells at a distance (endocrine)

The ability of cytokines to exert their control on target cells depends on their binding to specific high affinity, cell surface receptors. Once bound to their receptors, cytokines trigger intracellular signal cascades, which determine the nature of the immune response. Some responses of cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation and secretion of effector molecules [42]. These events have also been demonstrated in the superfamily of small structurally-related cytokines, termed chemokines. Chemokines are members of the α-subfamily of chemoattractant cytokines and play a major role in guiding lymphocytes and inflammatory cells to tissue sites [43]. They respond by determining which cells will cross the endothelium, and where they will move within the tissue [44].

Although cytokines are made by many cell populations, their predominate producers are T helper cells and macrophages. Cytokines have a pleiotropic nature and are extremely potent at low (picomolar) concentrations [2]. The largest group of cytokines are associated with stimulating immune cell proliferation and differentiation [42]. Members belonging to this group include IL-1, which activates T cells; IL-2, which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5 and IL-6,

stimulating proliferation and differentiation of B cells; IFN-γ activates macrophages; and IL-3, IL-7 and GM-CSF, which stimulates hematopoiesis [42].

The multi-functional nature of cytokines result from their expression of cognate receptors by different target cell types, each having distinct functions both within and outside the immune system. Many cytokines act through active multi-subunit receptor complexes that are frequently comprised of homodimer or heterodimer polypeptide subunits. Cytokines are divided into distinct groups depending their structure and sequence similarities of their cell-surface receptors. These are grouped into receptor families.

1.2.1.6.1 Type I receptor family

The largest family of cytokine receptors, the Hematopoietin family or Type I receptor superfamily is characterized by an extracellular region of structural homology approx. 200 amino acids (aa) long. Generally this receptor has 2 subunits, one cytokine-specific and one signal transducing [42]. Receptors of cytokines belonging to this family include IL-2, IL-3, IL-4, IL-5, IL-9 and GM-CSF [45].

1.2.1.6.2 Type II receptor family

This family of related cytokine receptors form part of the immunoglobulin superfamily. Receptors of all IFN types, as well as IL-10 and macrophage colony-stimulating factor (M-CSF) belong to this family.

1.2.1.6.3 Type III receptor family

Expressed on all nucleated cells and referred to as the TNF receptor family, these receptors all have multiple cysteine-rich repeats of about 40aa in their extracellular domain. In addition, to receptors for TNF-α and TNF-β (lymphotoxin) this family includes important molecules such as CD40 (important for B cell and macrophage activation, CD27 and the cell death signaling receptors Fas/CD95, DR4 and DR5) [42,45]. Members of this family include receptors for IL-8, MIP-1 and RANTES.

1.2.1.6.4 Type IV receptor family

Representatives of this family include IL-1α and β. The primary structure of these receptors shows little similarity, although both interact with the same cell surface receptors [46]. Two subtypes of IL-1 receptors have been identified: a type I (80kDa) receptor predominately found on T cells, and a type II (60kDa) receptor expressed primarily on B cells and myeloid cells [47]. The aa sequence of both receptors contain 3 immunoglobulin-like motifs in their extracellular domains, categorizing these receptors into the Ig gene superfamily [48,49]. The motifs share a common 3D structure and are thought to be involved in ligand binding. The 2 receptors differ considerably in their cytoplasmic domains [49].

1.2.1.6.5 Chemokine receptors

Chemokines bind to a class of receptors known as 7 transmembrane domain proteins. These are coupled to G nucleotide-binding proteins that cause cell activation. A few chemokine receptors are specific recognizing and binding only a single ligand, while others respond to several ligands [45]. Members of this family include CXCR3, CCR3, CCR4, CCR5 and CCR8.

1.2.2. Parasites

1.2.2.1 Parasite classification

A symbiotic relationship may be distinguished on whether the association between the organisms involved is detrimental to one of the two partners. Commensalism defines a relationship that is beneficial to one partner and at least not disadvantageous to the other [50]. Mutualism, a specialized type of commensalisms describes a relationship beneficial to both organisms. On the other hand, parasitism describes a relationship in which one animal, the host, is to some degree injured through activities of another animal, the parasite.

Parasites of humans are classified within the kingdom Animalia, which are separated into two subkingdoms, Protozoa and Metazoa. Protozoa comprises animals in which all life functions occur in a single cell, whereas Metazoa are multicellular animals in which life functions occur in cellular structures organized as tissue and organ systems. The animal parasites of human and most vertebrates are contained in several major subdivisions or phyla. Those containing organisms that parasitize man include the Protozoan subgroups; Sarcomastigophora, Ciliophora, Apicomplexa, Microspora; and the Metazoan subgroups; Platyhelminthes, Aschelminthes and Arthropoda [50]. Their classification is based on factors pertaining to their mode of reproduction and type of locomotive organelle, as well as their morphology of intra-cytoplasmic structures such as the nucleus.

1.2.2.1.1 Phylum Sarcomastigophora

This phylum is further divided into two subphyla: the Mastigophora or flagellates, and Sarcodina or amoebae. Reproduction in these organisms is asexual.

1.2.2.1.1.1 Mastigophora

Mastigophora move by means of specialized structures known as flagella. A flagellum is a long, thread-like extension of cytoplasm that functions by propelling

the organism through fluid environments. Examples of clinically significant flagellates include *Giardia lamblia*, *Dientamoeba fragilis* and *Trichomonas vaginalis* [51]. Diseases produced by these organisms are primarily the result of mechanical irritation and inflammation [50].

1.2.2.1.1.2 Sarcodina

The unicellular microorganisms belonging to this subphyla move by means of cytoplasmic protrusions called pseudopodia. Their lifecycle is relatively simple and divided into 2 stages: the active motile feeding stage (trophozoite) and the dormant, resistant stage (cyst) [51]. With the exception of the pathogenic *Entamoeba histolytica*, most amoebae found in humans are commensals [50].

1.2.2.1.2 Phylum Ciliophora

The ciliate group includes a variety of free-living and symbiotic species, with only the intestinal protozoan *Balantidium coli* pathogenic to humans. Disease produced by *B. coli* is a result of the organism's elaborate proteolytic and cytotoxic substances that mediate tissue invasion and intestinal ulceration [51]. Locomotion in ciliates is accomplished by cilia, relatively short threads of cytoplasm arising from small basal granules[50].

1.2.2.1.3 Phylum Apicomplexa

Members of this phylum are tissue parasites, which have complex lifecycles with alternating sexual and asexual generations. Species belonging to this group include 4 species of the blood parasites *Plasmodium* which cause malaria, species of *Isospora*, *Cryptosporidim*, and *Sarcocystis* parasitic in the mucosa of the intestinal tract [50]. Also belonging to this group is *Toxoplasma gondii*, a parasite detrimental during pregnancy. Toxoplasmosis within the first trimester results in spontaneous abortion, stillbirth or severe disease [52].

1.2.2.1.4 Phylum Microspora

These organisms are obligate intracellular pathogens. Microsporidia can be considered to be opportunistic parasites as they rarely cause disease in immunocompetent persons, but may do so with greater frequency in immunosuppressed individuals. The parasites are characterized by the structure of their spores, which have a complex tubular extrusion mechanism used for injecting infective (sporoplasma) material into cells [52]. Reproduction occurs by binary or multiple fission and by a process culminating in spore development.

1.2.2.1.5 Phylum Platyhelminthes

The Platyhelminthes, or flatworms, are multicellular organisms characterized by a flat, bilaterally symmetric body [50]. With the exception of Schistosomes, flatworms are all hermaphroditic, having both male and female reproductive organs in the same individual. Most members of this phylum are symbionts, while free-living species belong to the class *Turbellaria*. Organisms belonging to the classes Trematoda and Cestoda are parasitic.

1.2.2.1.5.1. Trematoda

Trematoda or flukes are generally flat, fleshy, leaf-shaped worms. They are equipped with two muscular suckers: an oral type, which is the beginning of an incomplete digestive system, and a ventral sucker, which is simply an organ of attachment [53]. Flukes have complex lifecycles with at least one intermediate molluscan host (snails and clams). Schistosomes belonging to this class account for some 200 million infections worldwide with *Schistosoma mansoni* the most widespread in Africa. Intestinal schistosomiasis associated with this species, induce hepatic and intestinal abnormalities. Eggs deposited in the bowel mucosa results in inflammation and thickening of the bowel wall accompanied with abdominal pain, diarrhea, and blood in the stool. Chronic infection may result in eggs being deposited in the spinal cord and brain causing severe neurological problems.

1.2.2.1.5.2 Cestoda

Members of this class have elongated, ribbon-like, segmented bodies, and their heads are equipped with a specialized attachment organ, the scolex. They have no digestive tract, absorbing food from their host's intestine through their soft body walls. Cestodes or tapeworms are all hermaphrodites. As adults, they inhabit the human intestine and have complex lifecycles involving intermediate hosts [53]. Some medically important cestodes include; *Taenia solium*, *Echinococcus granulosus*, *E. multilocularis* and *Hymenolepsis nana.*

1.2.2.1.6 Phylum Arthropoda

Arthropods are segmented and bilaterally symmetrical animals with a body well protected by a chitinous exoskeleton bearing paired, jointed appendages. Their digestive system is well developed, and their sexes are separate. Members of this group have been responsible for some of the most devastating plagues and famines known to mankind yet other species are essential for our existence providing us with food, clothing, medicines and protection of harmful organisms [54]. Classes that are of medical importance include;

1.2.2.1.6.1 Arachnida

These spider-like animals have bodies divided into two parts, the cephalothorax and abdomen. Scorpions, spiders, ticks and mites are all included in this class. Ticks and mites may transmit disease.

1.2.2.1.6.1.1 Ticks

As disease transmitting arthropods, ticks are efficient vectors of bacteria, viruses, rickettsiae, and protozoans, which they acquire when feeding on small mammals or birds [55]. Major tick-borne diseases include Lyme borreliosis, and ehrlichiosis. The site of the tick bite is manifested with erythema migrans (rash). In chronic infections, patients may experience oligoarthritis (juvenile arthritis), debilitating dysfunction of their central and/or peripheral nervous system, and/or persistent inflammatory skin disorders [56].

1.2.2.1.6.1.2 Mites

Scabies mites (Sarcoptes scabiei) infest human skin causing severe itching and sometimes a generalized rash [55]. They burrow under the skin, leaving tiny open sores and linear serpiginous burros (tracts) filled with mites and their eggs. Transmission occur by close human-to-human contact with infected persons [55].

1.2.2.1.6.2 Insecta

Considered the most important of the arthropods, insects have three pairs of legs and a body divided into three distinct parts: head, thorax and abdomen. Mosquitoes, flies, beetles, ants, and bees are among a few belonging to this class. They may be directly involved in causing invasive disease processes or indirectly as intermediate hosts and vectors of protozoan and metazoan parasites [57].

1.2.2.1.6.2.1 Mosquitoes

Mosiquito-borne diseases include malaria, yellow fever, dengue fever, filarisis, and numerous encephalitis viruses. The most prominent of these, malaria is caused by an infection with *Plasmodium falciparum*, *P. vivax*, *P. malariae* or *P. ovale* all of which are transmitted to humans by the female mosquito of the Anopheles genus. The initial symptoms of the disease are non-specific and may start with headache, fatigue, abdominal discomfort and muscle ache followed by fever. Other symptoms include seizures, anaemia and enlargement of spleen and liver.

1.2.2.1.6.2.2 Sandfly

These insects are significant vectors of the disease agents of leishmaniasis, bartonellosis, and sandfly fever. *Leishmania* transmitted by the female sand flies of the genus Lutzomyia (America) and Phlebotomus (elsewhere) infect macrophages. Leishmaniasis are divided into 3 major clinical syndromes: cutaneous, mucosal, and visceral [58].

1.2.2.1.6.2.3 Tsetse fly

Human African trypanosomiasis, or sleeping sickness, is caused by two morphologically identical subspecies of trypanosomes – Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense transmitted to humans by tsetse flies

[59]. Acute symptoms include fever, headache, myalgia (muscle pain), and malaise (general state of discomfort). Chronic disease symptoms include somnolence and severe headache. If untreated the disease may progress to stupor and coma and ultimately death [59].

1.2.2.1.7 Phylum Aschelminthes

1.2.2.1.7.1 Nematode

Nematodes, or roundworms belonging to the class Nematoda, are the most easily recognizable form of intestinal parasites because of their large size and cylindrical unsegmented bodies. They possess a stiff cuticle, which may be smooth or may be extended to form a variety of structures at the anterior and posterior ends. Parasites live primarily as adult worms of which a large number of species parasitize humans, animals, and plants.

Gastrointestinal nematodes, soil-transmitted helminths are amongst the most prevalent worldwide with an estimated 3.5 billion cases, of which 450 million are individuals who are seriously ill as a result of chronic disease. Children account for the majority of these cases and of the estimated worldwide numbers, 44 million are pregnant women infected with hookworms [60]. Of the 342 helminth species that infect humans [61], the majority of which remain asymptomatic, the most importance species of medical significance are *Ascaris lumbricoides* (roundworms), *Ancylostoma duodenale* and *Necator americanus* (hookworms), *Trichuris trichiura* (whipworm), *Enterobius vermicularis* (pinworm) and *Strongyloides stercoralis* (threadworm) [62]. The majority of people infected live in the developing world, with those living in rural areas and urban slums showing high risk most likely due to their environment. These poverty strictened areas are overcrowded with poor housing and living conditions, a lack of adequate sanitation and hygiene, as well as poor education and health care facilities. In addition, warm and humid climatic conditions as are found in the tropics and sub-tropics favor GI nematode survival with the exception of *E. vermicularis* which prefer low temperatures and high humidity as observed in the Northern hemisphere.

The six GI nematodes of major medically importance, all have direct lifecycles and are highly specific to humans with no animal reservoirs of infection for any of the species. With the exception of *E. vermicularis*, eggs and larva of the major nematodes all require a period of development in soil to become infective before transmission to humans [62]. This requirement, combined with the similar geographical distribution of GI nematodes, explain the high frequency of concurrent infections with multiple species. Despite being asymptomatic at low worm burdens multiple infections can exacerbate worm pathology [63].

The lifecycles of the major GI nematodes of humans are basically similar, although differences do exist. Adult worms reproduce sexually and mature females produce and release eggs into their immediate environment of the human intestine. From here, eggs are usually passed into the external environment via human feces leading to the development of L1 larvae within the eggs. Although difference in subsequent development follows, most species' L1 develop through four further stages (L2, L3, L4, pre-adult) with each stage preceded by a cuticular moult, and each moult causing an increase in parasite size. However, difference as to where and when these moults occur is species dependent. Similarly, the pre-adult worm development to mature adults, both within the host and the time for maturation to full stage fecund (egg laying) females differ between nematode species [63].

Common complains with regard to heavy GI nematode infections include diarrhea, abdominal pain and in the case of *A. lumbricoides*, obstruction of the gut [64-67]. More serious consequences of these types of infections include anaemia and malnutrition.

Anaemia is a typical concern in hookworm infections, but heavy *T. trichiura* infections have also been shown to cause iron-deficiency anaemia [68]. Hookworm-induced anaemia is most prevalent in people living in rural areas in developing countries, where agricultural labour is their main source of income. The disease reduces the individual's physical ability to carry out work associated with this way of life, leading to poor nutrition and increased hookworm infection [69] The extent of anaemia is dependent on factors such as the intensity of infection, the infecting nematode (*A. duodenale* causes much greater blood loss than *N. americanus*), and the

level of iron intake and host nutritional intake [70]. Pregnant women infected with hookworms are most at risk due to the severity of iron-deficiency anaemia that may increase their risk of death to themselves or their unborn foetus [71].

Malnutrition of livestock has been shown to vary with their control of GI nematode infections as the severity of infection is dependent on the nutritional status of the animal [72]. This is believed similar for humans infected with GI nematodes. Children suffering from malnutrition are vulnerable to problems associated with GI nematode infections [73], leading to continual loss of appetite as well as affecting their physical, cognitive and social development [69,74]. Malnutrition is further exacerbated after GI nematode infections as the nematodes damage the intestinal mucosal epithelial cells whilst feeding, resulting in the poor nutrient absorption by the host and leading to stunted growth [75].

The re-emergence of other serious diseases as a result of GI nematode infections is particularly important in developing countries. A protective Th1 immune response elicited to control tuberculosis (TB) and human immunodeficiency virus (HIV) is downregulated by helminth infections [62]. The induction of T regulatory cytokines, IL-10 and TGF-β produced by helminth infections are immunosuppressive and believed to inhibit immune response that protect against TB [62]. In addition, the downregulation of the immune response is believed to be responsible for the rapid progression of HIV to AIDS in the developing world [76-78]. Individuals with helminth infections show a marked Th2 immune response and are chronically immune-activated which increases their susceptibility to secondary infections requiring a Th1 immune response for protection. Important, is that HIV and TB vaccine candidates in endemic areas of helminthic infections might response poorly as a result of their dominant Th2 immune response [79,80].

1.2.2.2 Parasite immunology

Parasites have evolved over millions of years to become well adapted to their hosts. The host specific relationships, which they have acquired, have enabled them to avoid immune defense mechanisms by either exploiting or interfering with key mediators. They have established themselves in virtually every type of niche and are a major medical problem, particularly in subtropical and tropical regions worldwide [1]. Many of their complicated lifecycles have been known for years, but it is only recently, through the understanding of dominant mediators, (regulating the immune system), that host interactions have been elucidated. A few of the niches occupied in human hosts include the gut (e.g. amoebae, *Nippostrongylus*), bloodstream (e.g. African trypanosomes), within erythrocytes (e.g. *Plasmodium* spp.), in macrophages (e.g. *Leishmania* spp., *Toxoplasma gondii*), and muscle (e.g. *Trypanosoma cruzi*).

The infectious organisms have an enormous species diversity that further complicates their mode of infection. During their lifecycle they often migrate through various parts of the host's body, passing through different stages of development in different tissues, before finally reaching their maturation site. Some species can also change their surface antigen with each developmental stage, allowing stage specific immunity. An example of such a response can be observed with the protein coat of the sporozoite (the infective stage of the malarial parasite transmitted by the mosquito), which induces the production of antibodies that do not recognize the erythrocytic stage [1]. A similar scenario can be seen with trypanosomes (cause sleeping sickness) that constantly change their surface glycoproteins [1].

Some intracellular protozoa avoid the immune system by using cellular molecules and pathways to direct their entry into target cells. The merozite, an invasive form of the blood stage of malarial parasites binds certain receptors on the surface of the erythrocyte (red blood cell) and uses specialized organelles, the rhoptry, to enter host cells. The rhoptry is a specialized organelle for invasion of host cells and secretes enzymes to destabilize the host membrane. Rhopties are found only in Apicomplexa

(*Plasmodium*, *Babesia*, *Neospora*), not in the Sarcomastigophora (*Leishmania*, *Trypanosoma*) [81].

Gastrointestinal worms infect a third of the world population with severity of disease dependent upon the worm burden [82]. However, in children even moderate intensities of infection may be associated with stunt growth and slow mental development.

1.2.2.2.1 Immunity to gastrointestinal (GI) nematodes

Most helminths infect their host via the oral route, and live either at the mucosal surface of the gastrointestinal tract (GIT), or transverse this mucosal barrier on their way to their preferred niche [83]. In responds to an invading nematode, the gut-associated lymphoid tissue (GALT, lymphoid tissue associated with surfaces lining the intestinal tract) process antigen released by the parasite and initiates a cascade of specialized immune responses [8]

1.2.2.2.1.1 Innate immunity to GI nematodes

The innate immune system provides the first line of immune defense by non-specifically detecting the immediate presence and nature of an infection [84,85]. Gastrointestinal nematodes are deterred from invading the underlying mucosal epithelium by a thick mucus blanket. The mucus layer providing a formidable barrier between the mucosal epithelium and lumen of the GIT comprises water and extensive polymers of secretary mucins [86]. In the intestine the layer is in a dynamic state which enables the host to eliminate adherent pathogens with sloughed off mucus during peristaltic movement and defecation [87]. Pattern recognition receptors (PRRs), which have evolved to recognize pathogen-associated molecular patterns (PAMPs) on invading pathogens also play a role in maintaining the epithelial barrier function [84,85]. Their immediate detection of microbes delay parasite establishment and allow the initiation of an adaptive immune response.

1.2.2.2.1.1.1 Gastrointestinal mucins

Gastrointestinal mucins are large glycoproteins classed on their location and structure: membrane bound (Muc 3) and secreted (Muc2) forms. They form the major components of mucus and compete for binding sites on the underlying epithelium [88-90], preventing attachment of pathogens to the mucosal surface [86]. Secreted mucins synthesized and secreted by goblet cells of the intestine and colon, and mucus cells in the stomach are rich in threonine, proline and/or serine residues. The abundance of these amino acids provides several attachment sites for *O*-linked oligosaccharides. These glycosylation patterns affect the physical properties of mucin, influencing its rigidity, protease resistance and gel-forming capability. Transient alterations in these patterns are directly related to the expulsion of *N. brasiliensis* within the rat model and were suggested to be a general protection mechanism against enteric pathogens [91]. Recently, the goblet cell-specific protein RELMβ/FIZZ2 was shown to be induced after exposure of 3 phylogenetically distinct GI nematode pathogens (*N. brasiliensis*, *Trichuris muris* and *T. spiralis*). The maximal expression of the protein correlated with the production of Th2 cytokines and host protection [92]. Furthermore, RELMβ was shown to bind components of the nematode chemosensory apparatus and inhibit chemotaxic function of *Strongyloides stercoralis*. The protein impaired co-ordination of worm movement [92].

1.2.2.2.1.1.2 Pattern recognition molecules

Once a pathogen overcomes the luminal mucin barrier, the innate immune system at the level of epithelium and lamina propria distinguishes invading pathogens by recognizing pathogen-associated molecular patterns via PRRs. Examples of human pattern recognition molecules include;

1.2.2.2.1.1.2.1 Collectins and ficolins

These secreted proteins bind oligosaccharide structures on the surface of microorganism, initiating the death of bound microbes by complement activation and phagocytosis. Collectin and ficolins both possess collagen-like sequences linked to the C-type carbohydrate-recognition domain (CRD) and fibrinogen *β*-gamma homology domain (FBG), respectively. The domains form trimeric clusters at the ends of collagen triple helices. Here, they mediate binding to the surface sugars of

pathogens [93] and recruit mannose-binding lectin (MBL)-associated serine proteases (MASPs). MASP is known to initiate complement activation by cleaving C3. C1q is also a collectin which activates complement when binding directly to *T. spiralis* [94] and *S. mansoni* [95]

1.2.2.2.1.1.2.2 Pentraxins

C-reactive protein (CRP) and serum amyloid protein (SAP) are secretary and membrane-associated pattern recognition molecules belonging to the pentraxin family. Both function as opsonsins upon binding phorylcholine and/or phosphorylethanolamine commonly found on the surface of nematodes, trematodes and cestodes [96]. CRP activates the classical complement pathway by binding to C1q, and participates in natural resistance to schistome infection [97].

1.2.2.2.1.1.2.3 C-type lectins

C-type or Ca^{2+}-dependent lectins expressed on macrophages and dendritic cells (DCs) have recently been shown to bind with high affinity to schistosomes [98]. These molecules mediate phagocytosis and antigen presentation [99]. C-type lectins can also modify Toll-like receptor-mediated activation of DCs and steer immune responses by altering cytokine responses [100].

1.2.2.2.1.1.2.4 Scavenger receptors

Scavenger receptors (SRs) are cell surface glycoproteins that bind low-density lipoprotein (LDL) and lipopolysaccharides (LPS) with high affinity. They are multi-domain transmembrane proteins that mediate killing of *S. mansoni* via host LDL bound to the worm's surface [101].

1.2.2.2.1.1.2.5 Complement receptors

Complement receptors particularly CR3, are ideal examples of pattern recognition receptors involved in the innate immune response [102]. Being multifunctional, they are involved in phagocyte adhesion, recognition, migration, activation and microbe elimination [103].

1.2.2.2.1.1.2.6 Toll-like receptors

Toll-like receptors (TLR) are type-1 transmembrane proteins with ecto-domains containing interspersed leucine-rich repeat motifs [104]. Presently ten TLRs' have been identified. TLR-2 recognizes and binds lysophosphatidylserine from *S. mansoni* triggering the development of fully mature DCs capable of inducing a Th2 response, characterized by elevated IL-10 levels [105].

1.2.2.2.1.2 Adaptive immunity to GI nematodes

1.2.2.2.1.2.1 Protective Th2 immune response

Resolution of GI nematode infections is associated with an induction of Th2 immune responses characterized by the cytokines IL-4, IL-5, IL-9 and IL-13. T helper 1 dominated responses associated with IL-12 and IFN-γ production leads to chronic infection [106,107]. The protective response mediated by the Th2 cytokine IL-4, the IL-4 receptor, or Stat-6 signaling has been well-documented for the clearance of *Heligomosomoides polygyrus*, *T. muris*, *T. spiralis* and *N. brasiliensis* from the murine host [108-113].

Interleukin-13 also plays an important role in providing resistance to *T. muris* [108] and is essential in the expulsion of *N. brasiliensis* [111]. IL-4 and IL-13 have related functions as immune regulators. Both cytokines bind two receptors (Fig. 1.4), IL-4 to the IL-4Rα chain consisting of the classical IL-4R (type 1 IL-4R) and the type 2 IL-4R (Zurawski, 1994 #101;Zurawski, 1995 #76;de Vries, 1998 #78;Hilton, 1996 #79) to which ligation to either activates at least 2 Janus kinase 1 (Jak1) dependent signaling pathways, which involve tyrosine phosphorylation of Stat-6 or IRS-2 (Insulin Receptor Substrate-2) [114-125]. In the type 1 IL-4R, IL-4Rα is paired with the common γc (also a component of IL-2, IL-7, IL-9 and IL-15 receptors) [126-128], while in the type 2 IL-4R, IL-4Rα is paired with IL-13Rα1 [121,126,129-131]. IL-13 binds IL-4Rα-IL-13Rα1 complex (Fig. 1.4) with higher affinity than it does IL-13Rα1 alone [115,129-133]. The second IL-13 binding chain, IL-13Rα2 selectively binds IL-13 with a much higher affinity than either the IL-4Rα-IL-13Rα1 complex or IL-13Rα1 alone. The function of this receptor is still unclear but may include attenuation of some IL-13 responses [120,134] One can conclude that differences in the relative cell expression of

the IL-4 and IL-13 receptors explains differences in responsiveness to T-and-B-lymphocytes and other bone marrow derived cells, such as macrophages and eosinophils. It could also explain why IL-13 may be more potent as a stimulus for smooth muscle and epithelial cells [123,135].

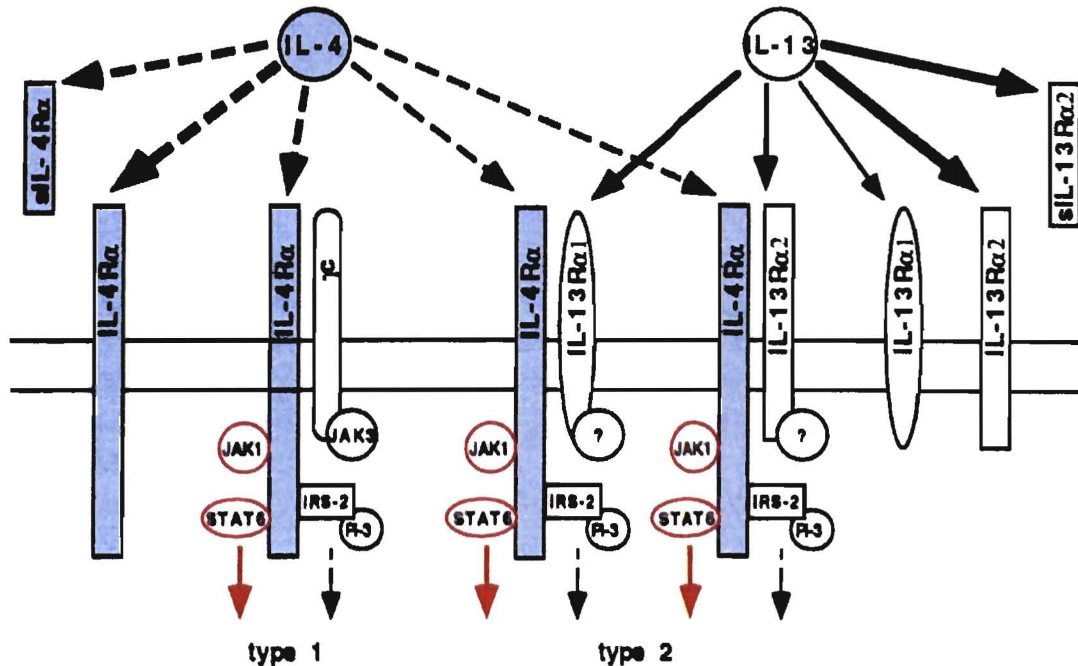


Figure 1.4: IL-4 and IL-13 receptor complexes. IL-4 interacts with the IL-4Rα binding protein in combination with either γc (IL-4 type 1 receptor), IL-13Rα1 or IL-13α2 (IL-4 type 2 receptor). IL-13 interacts with 13Rα1 or IL-13Rα2 binding proteins in combination with the IL-4Rα chain. Increasing thickness of arrows indicate increasing binding affinities. This figure was taken directly from Brombacher [136].

There are numerous ways in which IL-4 and IL-13 independently modify immune and inflammatory responses. These include; **(1)** enhancing monocyte/macrophage and endothelial cell expression of adhesion molecules [137]; **(2)** increasing macrophage expression of molecules associated with antigen presentation and T cell costimulation [115]; **(3)** stimulating the production of the chemokine, eotaxin [138]; **(4)** suppressing the production of inflammatory mediators such as prostaglandins [139], reactive nitrogen and oxygen intermediates [140,141] and; **(5)** stimulating the production of anti-inflammatory molecules such as the soluble IL-1Rα, the type 2 (decoy) IL-1R and the IL-1R antagonist [142-144]. In addition similarly as IL-4, IL-13 enhances the responsiveness of eosinophils to chemokines [145], suppresses IL-2 induced

proliferation of NK cells and their cytolytic activity [117] and stimulates human B cells to express CD23 and switch to expression of IgG4 and IgE [137].

IL-4Rα-deficient mice have phenotypic characteristics similar to those observed in the IL-4/13 double deficient mice [146]. Studies comparing the immune responses mounted by IL-4-deficient and IL-4Rα-deficient mice have provided evidence for the involvement of IL-13 receptor signaling in the generation of Th2 responses. This was well documented with *N. brasiliensis* infection where IL-4Rα responsiveness was required for protective immunity against the gut-dwelling nematode [146]. The response was independent of IL-4. Furthermore, IL-4Rα-deficient mice succumbed to chronic *Leishmania major* infection whereas IL-4-deficient mice control the infection, indicating that the functional IL-13 receptor protect mice from profound parasite induced pathology [39].

Recently, Brombacher and colleagues have generated macrophage, T cell and smooth muscle cell-specific IL-4Rα$^{-/-}$ mice respectively. These animals lack IL-4 and IL-13 responsiveness on the individual cell type and are used as a tool for understanding the relative importance of IL-4Rα signaling on the cell type and in disease.

1.2.2.2.1.3 GI nematode expulsion

Expulsion is mediated by a number of cellular types and is not restricted to immune cells alone. However, the immune system plays a crucial role in the induction of host protective responses to GI nematode infections. Mutant severe combined immuno-deficient (SCID) mice, lacking both T and B cells and Recombinase-Activating Gene (RAG)-2 mutant mice, lacking all lymphocytes, are resistant to helminths with the administration of IL-4 and IL-13 [146,147]. This demonstrates that non-lymphoid cells do contribute significantly to the protective immunity of GI nematodes. Possible non-lymphoid cells capable of a helminth-induced Th2 response include eosinophils, goblet cells, basophils and mast cells. Infection produces mucosal inflammation, and induces changes in intestinal physiology including enhanced intestinal propulsive activity, goblet cell hyperplasia, increased mucus secretion, and increased ion water secretion [148].

1.2.2.2.1.3.1 Eosinophils

Eosinophils are multi-functional cells capable of releasing potent cytotoxic and proinflammatory mediators in addition to expressing receptors for and secreting immunologically important molecules [149]. Both peripheral and tissue eosinophils are characteristic features of many helminth infections (Fig. 1.5). In mice, IL-5 controls the elevated rate of development, maturation and survival of eosinophils during a Th2 cytokine response [150]. Studies using IL-5 knock-out (KO) mice or anti-IL-5 antibody showed no change in the expulsion of a primary GI helminth infection [151,152]. However, *T. spiralis* expulsion was impaired in IL-5 KO mice upon a challenged infection [153]. The authors here suggested that IL-5 and eosinophils probably protect against repeated exposure to a GI parasite, such as *T. spiralis*. In schistosome infections, epidemiological studies have shown correlations between eosinophilia and protection [154,155]. Whether eosinophilia plays a role in the expulsion of GI nematodes remains debatable however, it has been suggested that these cells may represent an immunopathogical rather than protective response as a consequence of inflammation induced by a Th2 response to worm infections.

1.2.2.2.1.3.2 Mast cells

Mastocytosis is a known effector function creating an inhospitable environment important in the expulsion of *Strongyloides venezuelensis* and *T. spiralis* [156-160]. The cross-binding of IgE to FcεRI receptors on the cell surface of mast cells trigger the release of preformed and *de novo*-synthesized mediators, including histamine, prostaglandins, leukotrienes and proteases (granule *β*-chymase and mMCP-1, (Miller, 2002 #124). Subsequently these mediators result in increased GI motility, mucus hypersecretion, increased vascular permeability and leucocyte recruitment and activation. Other important roles of mast cells include their possible promotion of a Th2 response via the early production of IL-4 [161].

1.2.2.2.1.3.3 Smooth muscle cells

All muscle in the GI tract is smooth muscle with the exception of the first section of the oesophagus. Mucosal inflammation produced during the intestinal phase of *T. spiralis* infection is accompanied by increased intestinal muscle contractility and propulsive activity [162-164]. The response mediated by Th2 cytokines (IL-4 and IL-13)

via the Stat-6 pathway [165] results in accelerated intestinal transit. A similar IL-4/IL-13-dependent peristaltic movement was shown in mice infected with *N. brasiliensis* and *H. polygyrus* [166]. Furthermore, a role for IL-9 in intestinal muscle contractility has been demonstrated in *T. spiralis* infection [167]. These studies support the hypothesis that Th2 responses mediate muscle contractility in nematode infection.

1.2.2.2.1.3.4 Goblet cells

Goblet cells found throughout the small and large intestine are the main source of mucins in the gut [88]. Although the exact function of the mucus is not known, authors have suggested that it traps parasites in the lumen. The high levels of mucus deter parasites from anchoring in the gut, while leukotrienes and IL-4 increase gut motility [168]. Other factors like the recently described RELMβ (Resistin-Like Molecule) are also believed to affect the chemosensory apparatus of GI nematodes, impairing their survival [92].

The induction of hyperplasia has been described in a number of nematode infections including *N. brasiliensis* [89,169,170], *Strongyloides ratti* [171], *T. muris* [106,167] and *T. spiralis* [172,173]. However, a correlation between goblet cell hyperplasia and worm expulsion could only convincingly be shown with a *N. brasiliensis* infection [174].

1.2.2.2.1.3.5 Intestinal epithelial cell turnover

In a study using *T. muris*, the authors demonstrated accelerated intestinal epithelial cell turnover as an expulsion mechanism to displace worms from their optimal niche in the intestine [175]. Basically, the epithelium acts as an escalator whereby enterocytes migrate luminally, undergoing proliferation, differentiation, and maturation before undergoing programmed cell death and extrusion into the intestinal lumen [176]. Hence, the GI nematode *T. muris* is rapidly expelled together with its habitat. Resistance was shown to be IL-13 dependent. Susceptible mice demonstrated a delay in the acceleration of epithelial turnover upon which *T. muris* has quadrupled in length and undergone a further molt. The nematode now resided much higher in the crypt and was no longer in the compartment where the fastest movement of cells was occurring. Thus, susceptible mice became chronically infected harboring an increasing worm burden.

1.2.2.2.1.3.6 Antibody

The importance of an antibody response in the expulsion of GI helminths has been assigned in the control of a challenged rather than primary infection [105]. Gastrointestinal nematode infections induce high antibody production particularly Th2 associated IgG1 and IgE isotypes [177,178]. Most of the antibody produced in *T. spiralis* and *H. polygyrus* infections are non-specific, and in the case of *T. spiralis* are only produced after worm expulsion has been initiated. These studies suggest that antibody production is merely a consequence of a strong Th2 immune response [177,179].

Passive transfer of antibody (IgG and/or IgE) can enhance the expulsion of GI helminths however a lack of antibody does not hinder expulsion [177,180,181]. This was clearly demonstrated with *N. brasiliensis* and *T. muris* infections respectively, where the low level production [182,183] or lack of antibody [182] similarly had no effect on worm expulsion. In addition, Stat-6-deficient mice despite their high level production of antibody could not expel *N. brasiliensis* [111]. Similarly, FcγR-deficient mice showed normal expulsion of *T. muris* despite the lack of the IgE receptor [15].

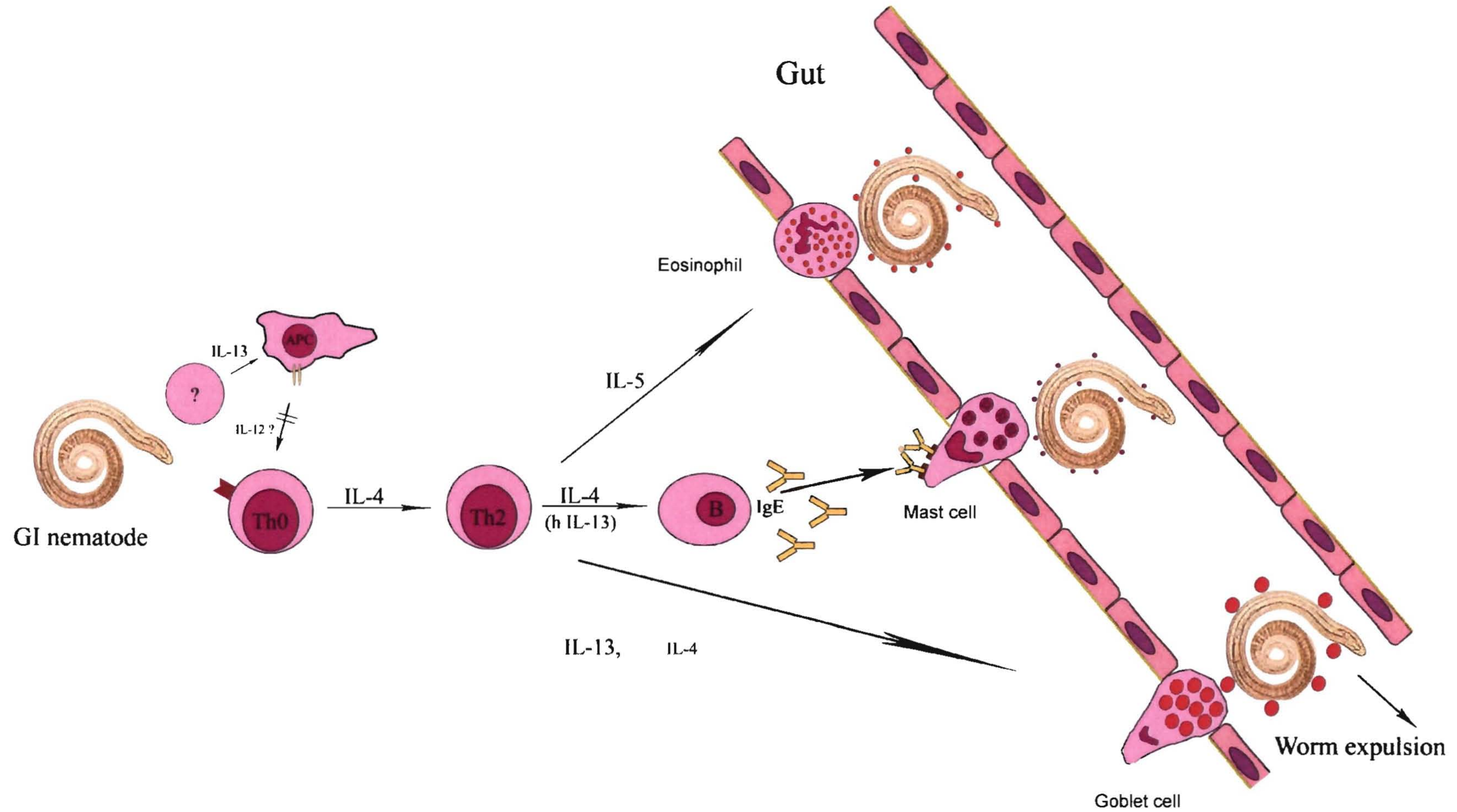


Figure 1.5: Proposed GI nematode expulsion model adapted from [100]. Role of Th2 cells, effector cells and cytokines in worm expulsion. Th0, naïve T helper cell; Th2, T helper 2 cell; B, plasma cell.

1.2.3 Pinworm

Enterobius vermicularis, human pinworm is one of the most prevalent intestinal nematodes of mankind [184]. Unlike other common intestinal nematodes, pinworm infections do not only affect individuals of tropical climates or populations with poor hygiene or low socioeconomic status. In addition, eggs deposited on the perianal region are immediately infective and do not require a period in the environment to embryonate. Transmission is largely from person to person and results in an irritating but not life-threatening perianal pruritus [184].

1.2.3.1 *Enterobius vermicularis*

Enterobius vermicularis is a nematode parasite most prevalent in the United States [67]. Presently, humans are the only known host with more than 200 million infected worldwide. Adult worms are relatively small with the males measuring 2 to 5mm and the females measuring 8 to 13mm. Male worms are less numerous, smaller in size with a curved tail, a small bursa like expansion, and a single spicule. In contrast, female worms boast a cuticular expansion at the anterior end leading to a long pointed tail. Adult worms live primarily in the cecum from where the gravid female (each containing up to 15000 eggs) migrates at night to the perineum and is stimulated by air contact to lay her eggs (Fig. 1.6).

The eggs are sticky and have a characteristic asymmetrical shape which is flattened on the one side. They measure approximately 55 by 30μm in size and mature to infectious stage (L1 larvae) before hatching in the duodenum [185]. During their maturation the larvae undergo a series of moults, as they migrate down the digestive tract. In the cecum the larvae mature into adults before copulating to complete their lifecycle (± 6 weeks, Fig1.6)

The majority of pinworm infections are asymptomatic although in some cases the emerging female and sticky masses of eggs cause perineal, perianal, and vaginal irritation [186]. As the females emerge at night, constant itching in an attempt to relieve

irritation could give rise to sleep disturbances. More serious disease can result although rarely, and include weight loss, urinary tract infection and appendicitis [187,188].

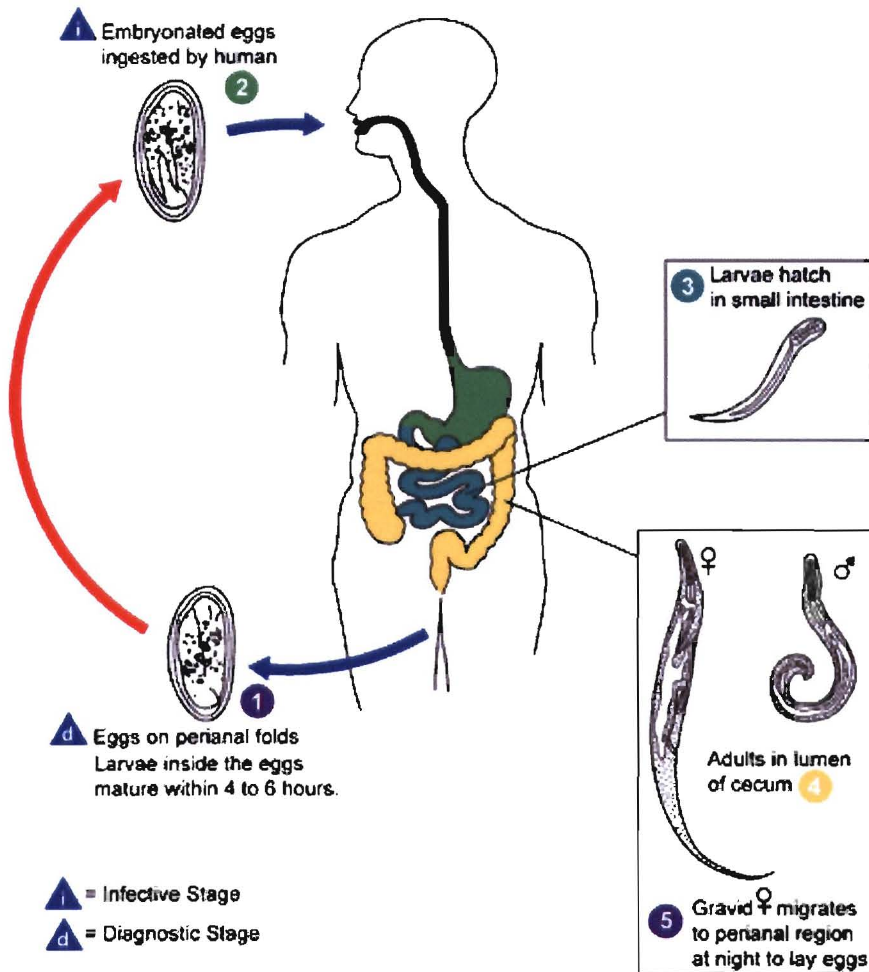


Figure 1.6: Pinworm lifecycle (*Enterobius vermicularis*). **(1)** Eggs deposited on the perineal folds are stimulated by O_2 to mature within 4-6hrs. **(2)** Eggs from perianal area or from contaminated food and water are ingested. **(3)** Larvae migrate to the ileum and molt twice. **(4-5)** Adults live in cecum and emerge nocturnally to lay eggs. This figure was taken directly from website of the Centre of Disease Control and Prevention [189].

1.2.3.1.1 *Enterobius vermicularis* as a probable vector for *Dientamoeba fragilis*

An intriguing example of pinworm acting as vector for protozoan parasites have been described in the case of *E. vermicularis* and *Dientamoeba fragilis*. *Dientamoeba fragilis* is a protozoan amoeboid organism that has for years escaped the interest of most clinical and diagnostic microbiologists [190]. The intestinal pathogen is not considered pathogenic in some countries as it has been exhibited in patients showing no apparent clinical symptoms [191] and is often identified in patients co-harboring other suspect pathogens. However, evidence supporting its pathogenicity has associated it as the cause of diarrhea, abdominal pain, flatulence, fatigue, and anorexia [192].

Humans are the only known natural host of *D. fragilis* and although most intestinal protozoa that are transmitted via the fecal-oral route require a cyst stage, none for *D. fragilis* have been identified in man. The cystic form of the organism protects it from the external environment as Dobell demonstrated it unlikely that the adult protozoa would survive their journey through the alimentary tract [193]. Dobell [193] hypothesed that *D. fragilis* is transmitted via the ova of nematodes and suggested *T. trichiura* and *Ascaris lumbricoides* as likely candidates. Burrows and Swerdlow found a 20-fold greater incidence of *D. fragilis* co-infection with *E. vermicularis*. They also observed small amoeboid bodies with similar nuclei to *D. fragilis*, in the eggs of pinworms [194]. The probability of pinworm as the intermediate host was further supported by Ockert [195,196] who successfully infected himself and two other human subjects, with *D. fragilis* by ingesting *E. vermicularis* eggs. However, the inability to identify a cyst stage of *D. fragilis* does not guarantee its non-existence and emphasizes the importance of establishing a suitable animal model to study the lifecycle and clinical symptoms of *D. fragilis*.

1.2.3.2 *Syphacia* species (rodent pinworm)

Syphacia species of the order, *Oxyurina* are GI nematodes primarily found in the cecum/rectum of their host (mouse, rat, hamster, gerbil, wild rodents) [197]. Because of its direct lifecycle, mice are infected by ingestion of the eggs through contaminated food, water and bedding. The gravid female deposits the elliptical-shape eggs at the perianal region of its host, which becomes infectious within 6hrs. At this stage it may follow one of 3 infectious pathways similar to that observed with the human species (Fig. 1.6).

(1) Direct pathway: the ingestion of embryonated eggs from the perianal region [198].

(2) Indirect pathway: the ingestion of water or food contaminated with embryonated eggs [198]; and

(3) Retro-infection pathway: when larvae from eggs that had hatched at the perianal region migrate back into the colon by way of the anus [199].

Syphacia obvelata completes its lifecycle within 11-15 days. The prevalence of pinworm in an infected rodent population depends on various factors such as;

(1) Age (increased resistance with advancing age of rats (Wagner, 1988 #165);

(2) Host immune status; and

(3) Sex, males are more parasitized than females [200,201].

1.2.3.2.1 Influence of pinworm on experimental diseases models

During the late 1960-1980's the general consensus was that pinworms were not pathogenic [197] and their inability to penetrate host intestinal tissue during development was believed to be the cause of no immune response being mounted by their host [202]. Today however, infection with *Syphacia* spp. continue to be a major global threat to science as rodents are directly infected by the ingestion of eggs through contaminated food, water and bedding. Despite strict regulations and drug treatments to eradicate this contagious parasite from experimental animals, re-infection continues to be a recurring problem, before and during research

experiments. More concerning is that this GI nematode possibly skew immune responses and effector mechanisms [203,204].

Beattie *et al.* [205], using a nude mouse-pinworm model system showed that *S. obvelata* induces the proliferation of T-and-B lymphocytes in the spleen, lymph nodes and occasional germinal centre formation. Sato *et al.* [204] followed up their research and showed for the first time a humoral immune response to pinworm infected mice. They also showed higher antibody production to non-parasitic antigenic stimuli, thus indicating that pinworm infections might modulate the immune system. Other influences associated with a pinworm infection include rectal prolapse, mucoid enteritis, and intestinal impaction/intussusception [206-212]. In addition, the gut dwelling nematode has been shown to influence the susceptibility of mice to other intestinal nematodes [203]. More recently, mice infected with pinworm were demonstrated to terminate self tolerance and enhance neonatal induction of a Th2-associated autoimmune disease and T cell memory [213].

1.2.4 Hypothsis

Gastrointestinal nematodes induce a Th2 immune response accompanied by IL-4, IL-5 and IL-13 cytokine production, eosinophilia mastocytosis, goblet cell hyperplasia as well as parasite-specific IgG1 and IgE antibody production. A similar protective immune response was hypothesized for the cecum-dwelling nematode, *Syphacia obvelata* isolated from one of our animal facilities. In this study we present the immune response elicited to a pinworm infection and demonstrate that IL-13 is the dominant cytokine required for the expulsion of the parasite. *S. obvelata* infection in BALB/c mice induced a protective response with elevated Th2 cytokines, and specific IgG1. In contrast, globally deficient IL-4Rα mice elicited a non-protective response to the worm with increased IFN-γ, parasite-specific IgG2b and a default Th2 response.

1.3 Material and Methods

1.3.1. Mice

IFN-γR$^{-/-}$ [214] and IL-12p40$^{-/-}$ [215] mice were generated on a 129/Sv/Ev background. IL-12p40$^{-/-}$ were backcrossed fives times to C57BL/6. IL-4$^{-/-}$ [216], IL-13$^{-/-}$ [217], IL-4/13$^{-/-}$ [218] and IL-4Rα$^{-/-}$ [39] mice were generated on a BALB/c background. All mice were kept at the animal facility at the Health Science faculty, University of Cape Town (UCT) under specific pathogen free conditions. In addition, mice were tested serologically for pinworm-specific IgG antibody prior to experiments. Eight to 12 week old mice were used for experiments performed in accordance with the guidelines of the Animal Ethics Research Board of UCT (Cape Town, South Africa).

1.3.2. Culturing of *S. obvelata*

1.3.2.1 Stahl's method

The cecum from susceptible mice strains (IL-13$^{-/-}$, IL-4/13$^{-/-}$, IL-4Rα$^{-/-}$) were collected in 0.65% NaCl (Merck, SA) and incubated in a gauze mesh at the mouth of a conical flask for 1-2hrs at 37°C. The worms were collected at the bottom of the flask and washed 3 times in 0.65% NaCl (1500rpm for 5min) before being processed for either eggs or antigen.

1.3.3. Egg and antigen preparation

1.3.3.1 Eggs

Worms collected from infected mice were crushed and debris removed through a 70μm cell strainer (Falcon, BD Biosciences, Belgium). The resulting contents containing the eggs were collected in a glass petri dish and washed in 0.65% NaCl (1500rpm for 5min) before being counted for infection.

1.3.3.2 Antigen

Isolated pinworms were equilibrated in a 40% (20ml Percoll in 30ml 0.65%NaCl) Percoll column at 2000rpm for 5min, which pelleted intestinal debris and suspended the worms. The suspended worms were transferred to a new 50ml Greiner tube and pelleted with dH_2O on the bench. To a volume of 10ml isolated worms 500-1000μl protease cocktail inhibitor was added and the solution sonicated at 17-19rms for ~1hr (1-2min sonicated intervals) on ice. The suspension was centrifuged at 1500rpm for 10min and the resultant supernatant filtered through a 0.2μm acetate syringe filter (Schleicher and Schuell, Microscience, Germany). The filtered protein was dialyzed (Spectrapor; MW 12 000-14 000, Sigma, USA) O/N in a cold room (4°C) and the concentration determined using a BCA kit [119]. Samples were stored at -80°C until use.

1.3.4. Infection

Eight to 12 week old mice were inoculated orally with 500 *S. obvelata* eggs using a 12G 20mm gavage tube (UCT, South Africa).

1.3.5. Antibody response

1.3.5.1 Collecting blood

1.3.5.1.1 Anesthesia with exsanguinations

Anesthetized mice were killed by exanguination (fatal blood loss). Mice were decapitated with a sharp pair of scissors and allowed to hemorrhage via the major cervical vasculature. Blood was collected and transferred into a serum separator tube (Becton Dickinson, USA).

1.3.5.1.2 Tail vein bleed

The mouse tail was heated under an infrared fluorescent lamp to dilate the veins. The tail was extended and an incision made across the lateral tail vein with a disposable scalpel blade (Aesculap, Germany). From the incised site blood was collected into a serum separator tube. Gentle pressure was then applied to the wound to ensure hemostasis.

1.3.5.2 Antibody ELISA

Antibody (Ab) responses were quantified by the highly specific and sensitive Enzyme-Linked Immunosorbent Assay (ELISA). The procedure involved coating microtitre plates with an antigen (Ag), which is bound by serum antibodies. The antigen-serum complex is then detected by a secondary Ab, specific for mouse immunoglobulin Ig's in addition to being labeled with an enzyme. The enzyme catalyzes the substrate to a product, which is chromogenic, it emits light that can be quantified at a particular absorbance depending on the substrate used.

Blood collected in serum separator tubes were centrifuged at 4100-4500rpm for 15-20min to separate the serum. IgG1, IgG2a, IgG2b, and IgE antibody isotypes were assayed from the serum using an indirect ELISA. For a specific antibody response 96-well microtitre plates (Nunc-immuno maxisorp surface plates, Denmark) were coated with crude soluble *S. obvelata* extract at 5μg/ml/50μl/well for 3hrs at 37°C and/or O/N at 4°C. For total IgE, plates were coated with anti-IgE antibody (84.1 C, 0.71mg/ml) diluted in coating buffer (Carbonate buffer, pH9, 0,02% NaN_3). Plates were then blocked with 2% milk powder (Spar brand, Spar, South Africa) at 37°C for 3hrs and/or O/N at 4°C. This was followed by the addition of serum in 10 fold dilutions starting with 1/10 and ending with 1/10^3. Serum samples were incubated at 37°C for 3hrs and/or at 4°C O/N. Plates were developed with anti-mouse isotype-specific polyclonal alkaline phosphate labeled antibodies (1/1000 IgG1, IgG2a, IgG2b, and IgE, Southern Biotechnology Associates, Inc), incubated for 2hrs at 37°C, followed by the addition of 50μl/well of alkaline phosphate substrate (Boehringer, Mannheim,

Germany). Naïve BALB/c serum was used as a negative control. The absorbance was read between 405 and 490nm by a Versamax turnable microplate reader (Molecular devices, USA).

1.3.6. Protein concentration (BCA kit)

A volume of 10μl standard or sample (diluent: 1×PBS, dH_2O or 0.65% NaCl) was added in duplicate to a 96-well flat bottom plate (Nunc, Denmark). This was followed by the addition of 200μl/well of BCA working solution (Pierce, USA) (50 parts solution A mixed with 1 part solution B) and an incubation of 30min at 37°C. The plate was allowed to cool to room temperature and read at an absorbance of 540nm (690nm reference) to determine the protein concentration.

1.3.7. Isolation of organs

Mice killed by cervical dislocation or exanguination were dipped in 70% ethanol (Merck, SA) and pinned to the dissection board. An incision in the lower abdomen, allowing the skin to be pulled back and exposing the internal organs was then performed. Aseptically, the spleen and draining lymph nodes were removed, divided and transferred to polypropylene test tubes (Grenier Bio-One, Germany) before being added to Ischov's media (Gibco, Life Technologies, USA) for cell restimulation (cytokines). For histology, intestine, colon, spleen, and lungs were removed and preserved in 4% formaldehyde solution (Merck, SA) and stored at RT.

1.3.8. Cell restimulation

Draining lymph node and spleen was aseptically removed and placed in ice-cold IMDM media supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin (Gibco, Life Technologies, USA). The organs were teased through a 70μm nylon cell strainer into a bacterial graded petri dish (Sterilin, UK), transferred to a 50ml Greiner tube (Cellstar, Grenier Bio-One, Germany) and centrifuged at 1200rpm for 8min (4°C). The pelleted cells were

resuspended in 5ml Red Cell Lysis Buffer and underlayed carefully with 2ml heat inactivated FCS before being centrifuged at 1200rpm for 8min (4°C). The cells were washed twice with media (1200rpm for 8min at 4°C), before being resuspended in 10ml media. Cells were diluted appropriately with trypan blue (Sigma, USA) and counted on a haemocytometer (0.1mm, Neubauer, Germany) to determine the cell concentration. For cytokine ELISAs 2×10^6 cells were cultured at 37°C and in 5% CO_2 in flat-bottomed 48 well plates (Corning, USA). Cells were restimulated with media, con A, 50 or 100μg/ml *S. obvelata*-Ag and α-CD3 in triplicate. The plates were incubated for 96hrs, supernatant collected and stored at -80°C for cytokine ELISAs.

1.3.9. Analysis of cytokine ELISAs

Cytokine ELISAs were performed to determine the Th cell immune response to pinworm. Th1 cytokines included IL-12 and IFN-γ. Th2 cytokines included IL-4, IL-5, IL-9, IL-10 and IL-13. The cytokines were detected by coating 96-well microtitre plates with 50μl/well of their respective cytokine coating antibody diluted 1/1000 (1-2μg/ml) in coating buffer and incubated for 2hrs at 37°C and/or 4°C O/N. The plates were then blocked with 200μl/well 2% milk powder at 37°C for 1hr and/or 4°C O/N. This was then followed by the addition of recombinant standard diluted 3-fold from 1-12 wells (working concentration, IL-4; 250ng/ml, IL-5; 250ng/ml, IL-9; 250ng/ml, IL-10; 100ng/ml, IL-12p40; 100ng/ml, IL-13; 100ng/ml and IFN-γ; 100ng/ml). Stimulated cytokine supernatant samples diluted 3 fold were added, 50μl/well and incubated for 37°C for 3hrs and/or 4°C O/N. Plates were developed with 50μl/well biotin labeled secondary Ab diluted 1/1000, except for IL-9 and IL-10 diluted 1/500, and incubated for 3hrs at 37°C. Then 50μl/well 1/1000 alkaline phosphatase or 1/10 000 horseradish peroxidase (HRP, only IL-10) labeled streptavidin was added and the plates incubated for an hour at 37°C, followed by the addition of 50μl/well of alkaline phospatase (AP) substrate (1mg/ml) or TMB peroxidase substrate (KPL, USA). The absorbances were read between 405 and 492nm for AP substrate and 450 and 540nm for TMB substrate (after stopping the rxn with phosphoric acid, HPO_3).

1.3.10. Eosinophilia

1.3.10.1 Peripheral blood eosinophilia

A volume of 10μl of blood collected was diluted 1/10 in Discombes fluid [219]. Cells were counted on a haemocytometer. The number of eosinophil cells/ml of blood was calculated using the following formula:

Number of cells $\times$ dilution factor (10) $\times$ 1 $\times$ 10^4

1.3.10.2 Eosinophil peroxidase (EPO) assay

10.2.1 Tissue isolation

Colon and intestinal tissue segments were aseptically removed, weighed and placed in Tris-triton buffer (pH8). The tissue samples were homogenized followed by a 30sec sonification at 18-20 watts. The resulting suspension was centrifuged at 1500-2000rpm and the supernatant aliquoted. Samples were stored at -20°C until use.

To a flat bottom 96-well plate, 75μl standard (HRP) and sample diluted serially 1/3 (0.5-2.8$\times 10^{-6}$ Units) in Tris-triton buffer was added. The plate was then incubated at 37°C for 5min. To each well was then added 75μl o-phenylenediamine (OPD, Sigma, USA). The reaction was stopped with phosphoric acid (Merck, South Africa) and eosinophil degranulation quantified at an absorbance of 490nm [220].

1.3.11. Intestinal mastocytosis

1.3.11.1 Mouse mast cell protease-1 assay

The levels of mouse mast cell protease 1 (mMCP-1) in serum and tissue homogenates (collected as for EPO) were measured by using a mMCP-1 ELISA kit purchased from Moredun Animal Health Ltd (Penicuik, United Kingdom) as previously described [221]. Briefly, rabbit anti-mMCP-1 was used as a capture antibody. Tenfold serial dilutions of serum or tissue homogenates were made from 1/10 to 1/10 000. Horseradish peroxidase-conjugated rabbit anti-mouse mMCP-1 was then added, and quantification was carried out by reference to purified mMCP-1.

1.3.12 Periodic Acid Schiff (PAS) staining technique for carbohydrates

1.3.12.1 Preparation of samples

Approximately 1cm of intestine and colon of naïve and infected mice were embedded in 4% formalin O/N.

1.3.12.2 Staining

Prepared sections were placed in dH_2O then oxidized in 1% aqueous periodic acid for 5-10min. This was followed by a 5min wash under running H_2O and rinse in dH_2O. The sections were treated with Schiff reagent for 15min followed by a 10min wash under running H_2O. Before being mounted, the sections were counterstained with haemotoxylin for 5min then dehydrated. PAS positive (bright red); Nuclei (blue); Other tissue (yellow).

1.3.13 Statistics

Data are given as mean ± SD, and the differences were tested using an unpaired Student's *t*-test or ANOVA.

1.4 Results

1.4.1. An outbreak of pinworm identified type 2 immune responses protective

Irregularities in some of our experimental results lead to the isolation and identification of a GI nematode in one of our specific parasite free (SPF) animal facilities. An outbreak of pinworm, later identified by its bulb-like esophageal morphology as *S. obvelata* was collected from the cecum of mice housed in this unit. Counting of worms showed that inbred BALB/c and C57BL/6 mouse strains could well control the infection with parasite loads below 50 worms (Fig. 1.7). No worms were observed in 129/Sv wild types, despite pinworm-specific anti-sera (Fig. 1.8E-H), which may indicate increased resistance in this mouse strain. Mice deficient for type 2 responses were chronically infected harboring approximately 1000 or more nematodes. Interestingly however, was the significantly low worm recovery from BALB/c IL-4$^{-/-}$ mice to that observed in the absence of IL-13 responsiveness (IL-13$^{-/-}$, IL-4/13$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice). Although these mice had fewer worms, the parasite load was significantly higher than the BALB/c controls suggesting that IL-4 plays a role in chronic pinworm infection (Fig. 1.7). In contrast, type 1 deficient mice, IFN-γR$^{-/-}$ and IL-12p40$^{-/-}$, were less susceptible with similar worm loads to that found in the inbred strains. The results here suggest that type 2 immune responses elicit protection against *S. obvelata* with roles for IL-4 and IL-13.

1.4.2. Isotype specific antibody response to mice naturally infected with *S. obvelata*

Sera from naturally infected mice were assayed by ELISA to establish the specific antibody isotype response to *S. obvelata*. IL-$4^{-/-}$ provoked a type 1 response with increased IgG2a and IgG2b and low levels of specific-IgG1 and total IgE when compared to the BALB/c wild type (Fig. 1.8A-D). BALB/c and IL-$13^{-/-}$ mice responded with a mixed type 1/type 2 antibody response. All 4 isotypes were elevated in these mice (Fig. 1.8A-D). C57BL/6 and IL-12p$40^{-/-}$ mice induced a type 1 antibody response with a marked increase in IgG2b production. These strains produced moderate levels of *S. obvelata*-specific IgG1, IgG2a and total IgE (Fig. 1.8E-H). 129/Sv and IFN-$\gamma R^{-/-}$ mice induced a mixed type1/type 2 responses with increased levels of specific-IgG2a and total IgE (Fig. 1.8E, H).

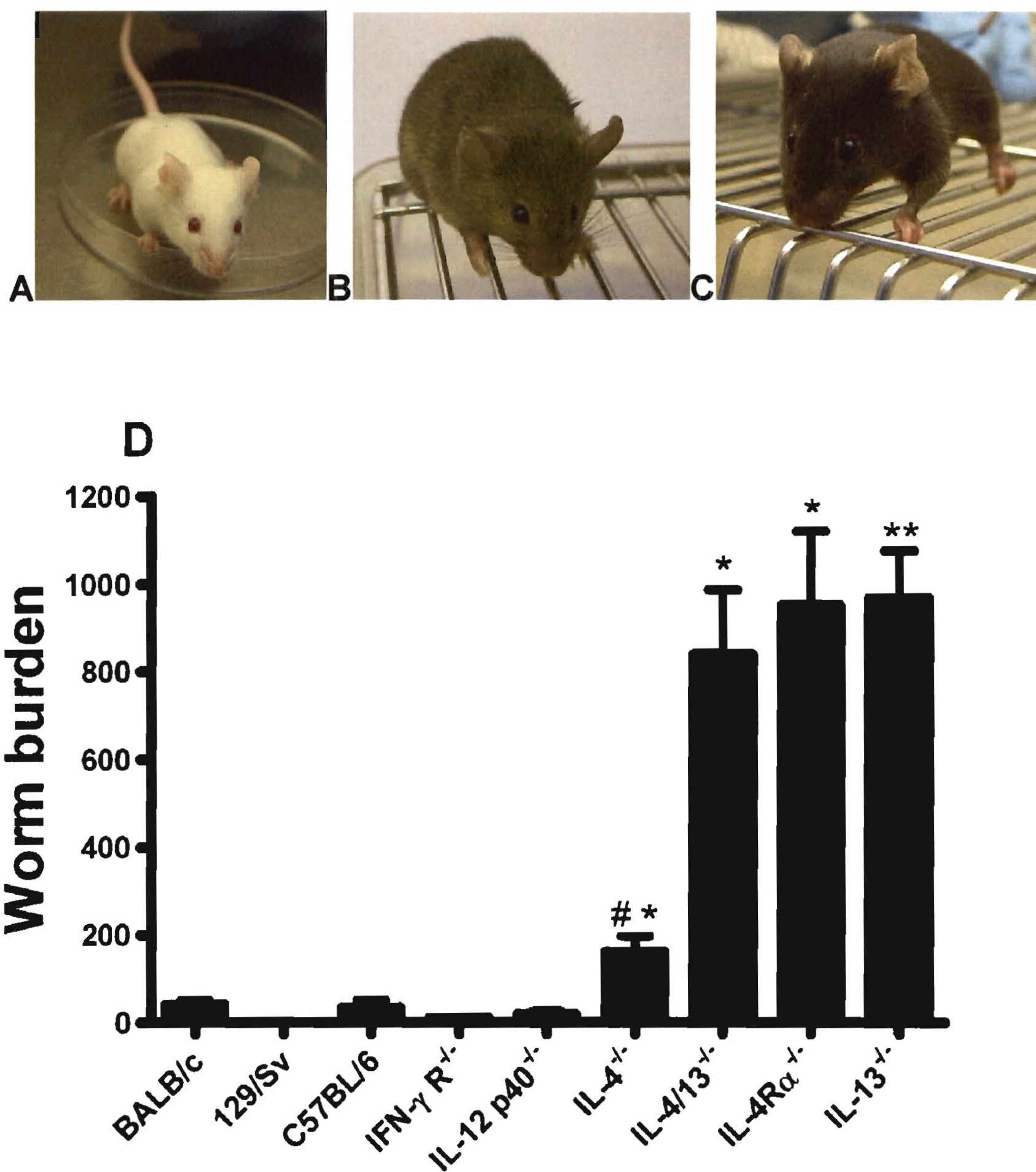


Figure 1.7. Naturally infected mice maintained in a conventional animal facility. **(A)** BALB/c, **(B)** 129/Sv and **(C)** C57BL/6 were the inbred mouse strains used in this study. **(D)** *Syphacia obvelata* susceptibility of naturally infected mice maintained in a conventional animal facility. Results are expressed as means of the number of worms found in the cecum. *, $p < 0.05$; **, $p < 0.01$, significantly different from BALB/c. In addition, the worm burden of IL-4 knockout mice were compared with those of highly susceptible mice: IL-$4^{-/-}$ versus IL-$13^{-/-}$, IL-$4/13^{-/-}$ and IL-4R$\alpha^{-/-}$ mice, #, $p < 0.05$. Four mice/group ± SD

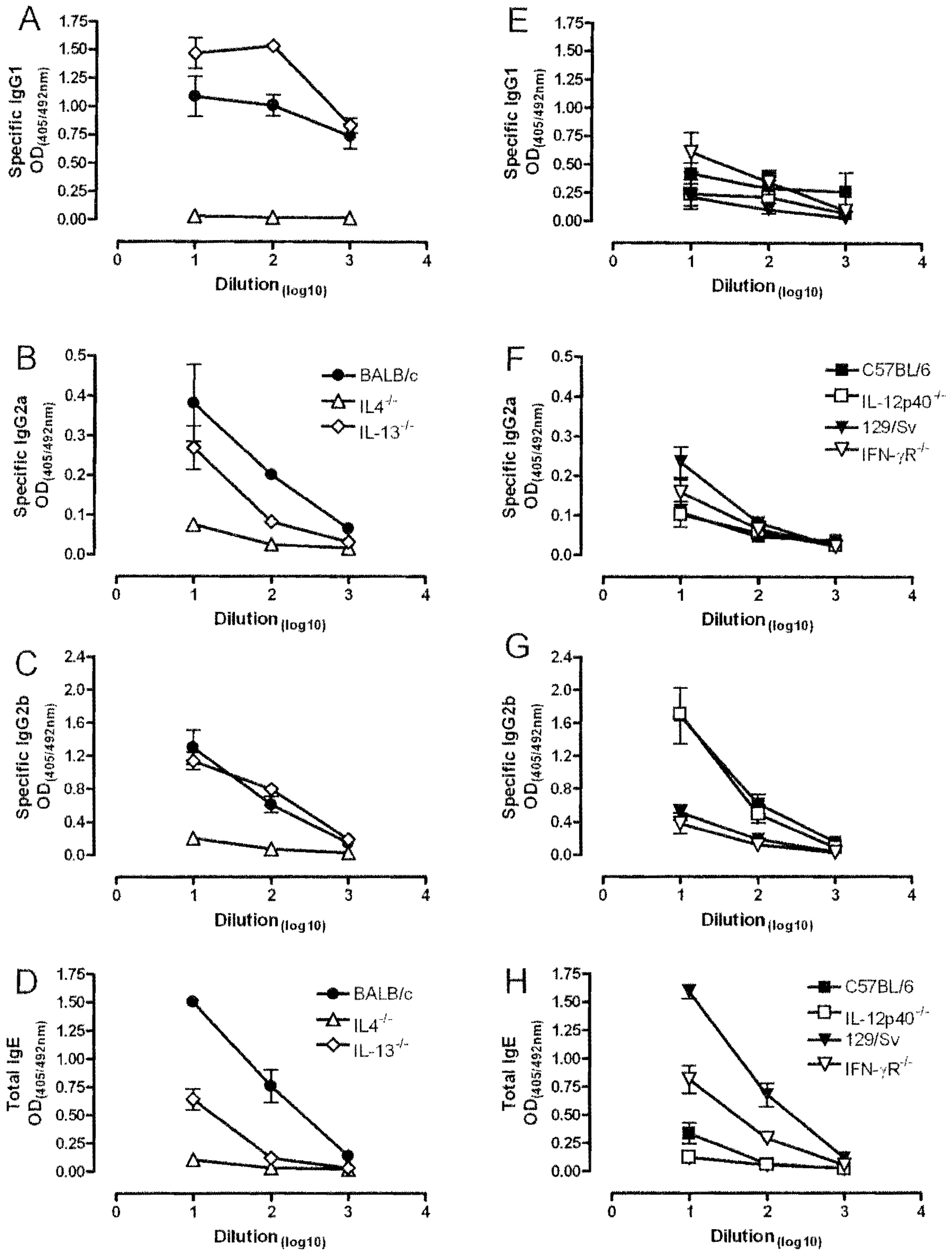


Figure 1.8. Antibody isotype profile. Sera from pinworm infected mice were assayed by an end-point titration ELISA to determine their antibody isotype response. Mice on different genetic backgrounds became naturally infected in one of our SPF animal units. Results are expressed as means of absorbance at 405/492nm. Four mice/group ± SD.

1.4.3. *Syphacia obvelata* resistance is IL-4Rα-dependent

The importance of IL-4 and IL-13 mediated functions in the expulsion of *S. obvelata* was investigated in an experimental infection model. BALB/c and IL-4Rα$^{-/-}$ mice inoculated with 500 *S. obvelata* eggs were killed weekly and their cecal contents analyzed. BALB/c mice induced a resistant phenotype evident by the low worm burden observed during a primary *S. obvelata* infection. The later worm recovery, day 35 postinfection (Fig. 1.9), possibly reflects a challenged infection as adult worms require 11-15 days to mature [198]. Conversely IL-4Rα$^{-/-}$ mice became chronically infected with significantly increased worm loads at day 28-35 postinfection. These mice were unable to expel the nematode and developed a chronic infection. Hence, we have demonstrated that resistance to *S. obvelata* is IL-4Rα-dependent since the absence of this receptor rendered mice highly susceptible.

1.4.4. Endogenous IL-13 and not IL-4 is the dominant cytokine eliciting protection

To closer dissect the roles of IL-4 and IL-13, gene-targeted mice deficient in IL-4, IL-13, IL-4Rα and double deficient IL-4/13 were infected with *S. obvelata* eggs and analyzed at day 35 postinfection. In the absence of the IL-13 cytokine (IL-13$^{-/-}$ and IL-4/13$^{-/-}$) and its responsiveness (IL-4Rα$^{-/-}$) mice became chronically infected with an uncontrollable parasite burden to that observed in wt (Fig. 1.10). In contrast, IL-4$^{-/-}$ mice demonstrated resistance with a similarly low worm load as observed in the BALB/c controls. The results shown here clearly demonstrate that IL-13 is the dominant cytokine eliciting protection against the GI nematode *S. obvelata*.

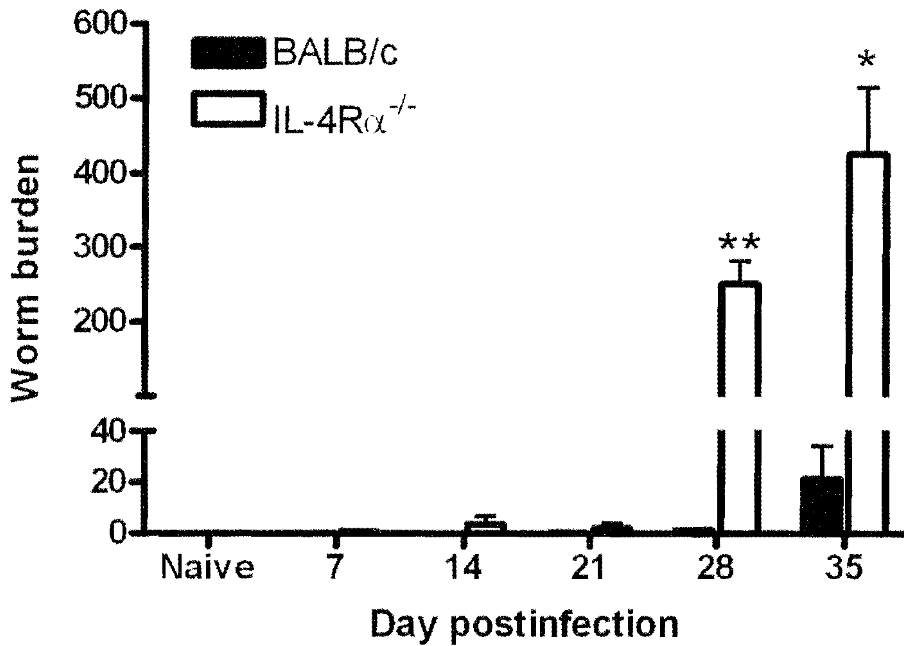


Figure 1.9. Worm burden of an experimental infectious model. Mice orally inoculated with 500 *S. obvelata* eggs were monitored weekly for worm burden. *, $p < 0.05$; **, $p < 0.01$, significantly different from BALB/c. One representative of two experiments is shown. Four mice/group ± SD

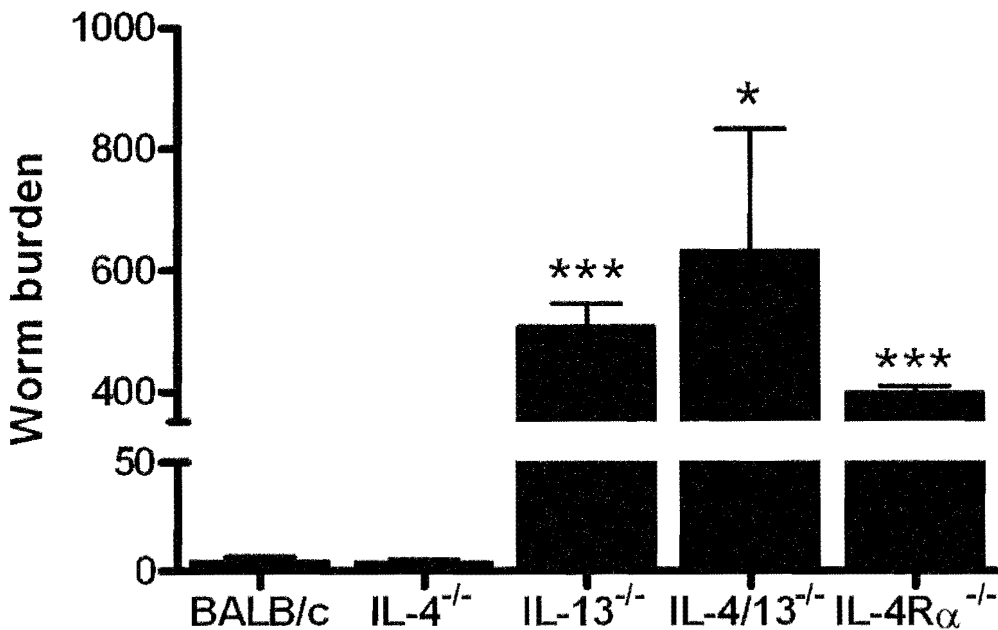


Figure 1.10. IL-4 and IL-13-mediated functions induce protective immunity to *S. obvelata*. BALB/c, IL-$4^{-/-}$, IL-$13^{-/-}$, IL-4/$13^{-/-}$ and IL-4R$\alpha^{-/-}$ mice were infected orally with 500 *S. obvelata* eggs and monitored 35 days postinfection. Results are expressed as means of worm burden. *, $p < 0.05$; ***, $p < 0.001$, significantly different from BALB/c. One representative of two experiments is shown. Four mice/group ± SD.

1.4.5. *S. obvelata* induces a Th2-type immune response

To investigate the Th immune response induced to *S. obvelata* we established the kinetic of cytokine production in the mesenteric lymph node (MLN) and spleen cells. Cells collected weekly were restimulated for 96hrs *in vitro* with anti-CD3, *S. obvelata* Ag and Con A and assayed by ELISA. Anti-CD3 stimulated splenic T cells from BALB/c mice produced a peaked cytokine response at day 7 postinfection which rapidly declined thereafter. These mice induced a Th2 response with significantly elevated IL-4 (Fig. 1.11B), IL-5 (Fig. 1.11C), IL-9 (Fig. 1.11D), IL-10 (Fig. 1.11E) and IL-13 (Fig. 1.11F) cytokine production. A transient IFN-γ (Fig. 1.10A) production was also evident at this time point. In contrast, IL-4Rα$^{-/-}$ mice provoked a Th1 response with an increase in IFN-γ production and lower secretion of Th2 cytokines. Surprisingly, Th responses in the MLN were lower (IL-4 and IFN-γ) and non-detectable (IL-5 and IL-13) to that observed systemically (data not shown). In addition, cells restimulated with 50 or 100μg/ml *S. obvelata* Ag produced lower cytokines responses suggesting that the nematode might modulate immune responses (data not shown).

Parasite-specific IgG isotype responses reflected the polarized Th response observed in BALB/c and IL-4Rα$^{-/-}$ mice respectively. BALB/c mice produced a mixed type 1/type 2 response with increased IgG1 (Fig. 1.12A) and IgG2b (Fig. 1.12B) antibody. Conversely, a type 1 immune response was demonstrated in IL-4Rα$^{-/-}$ mice with elevated IgG2b (Fig. 1.12B). Total IgE responses were not induced in either wild type or mutant mice (Fig. 1.12C).

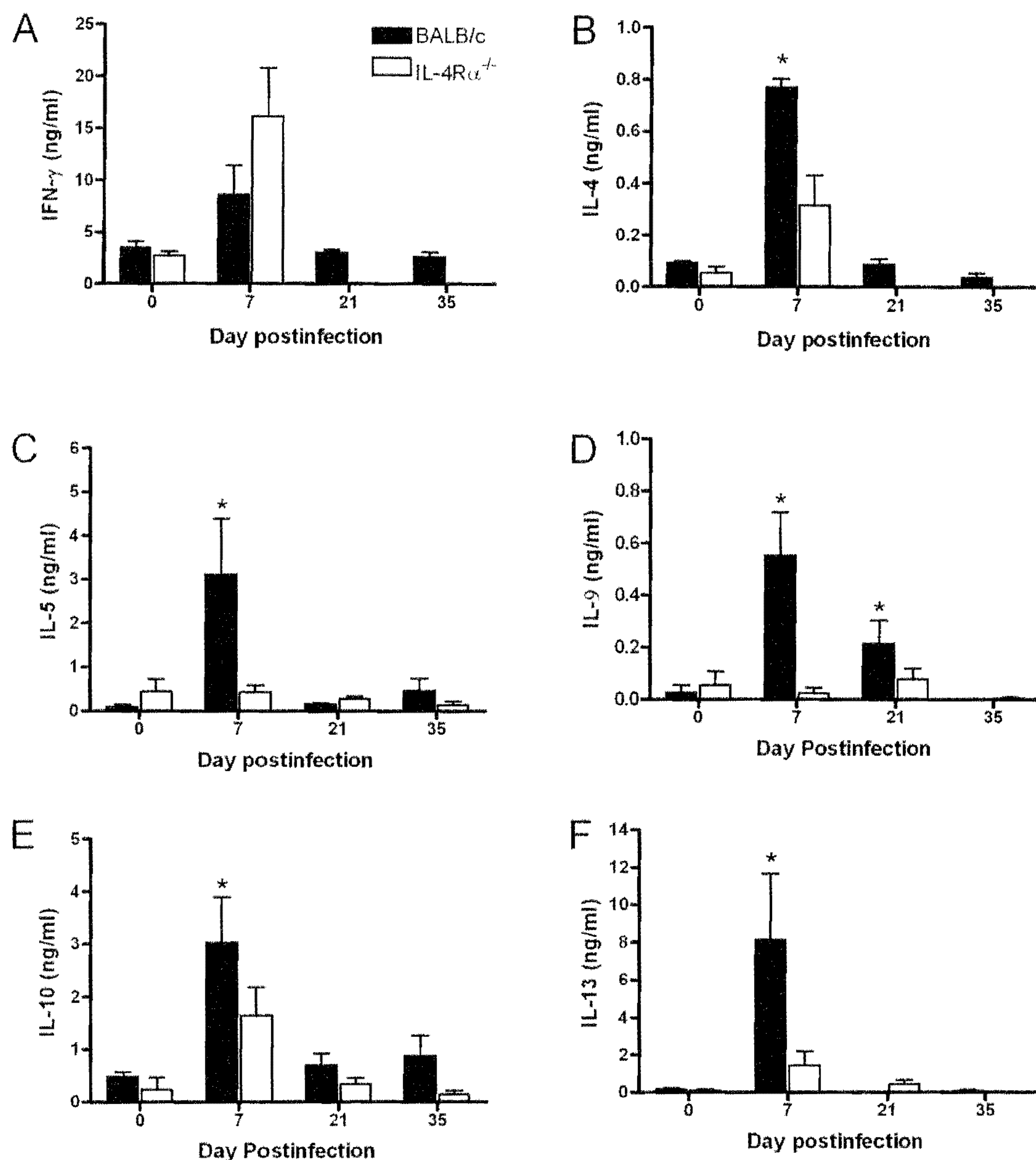


Figure 1.11. Immune response to helminth in BALB/c and IL-4Rα$^{-/-}$ mice. **(A)** IFN-γ, **(B)** IL-4, **(C)** IL-5, **(D)** IL-9, **(E)** IL-10 and **(F)** IL-13 production by anti-CD3 stimulated splenocytes from *S. obvelata* infected BALB/c and IL-4Rα$^{-/-}$ mice. *, $p < 0.05$, significantly different from naïve. One representative of two experiments is shown except for IL-9 and IL-10 which was only analyzed once. Four mice/group ± SD, naïve IL-4Rα$^{-/-}$, n = 2mice/group.

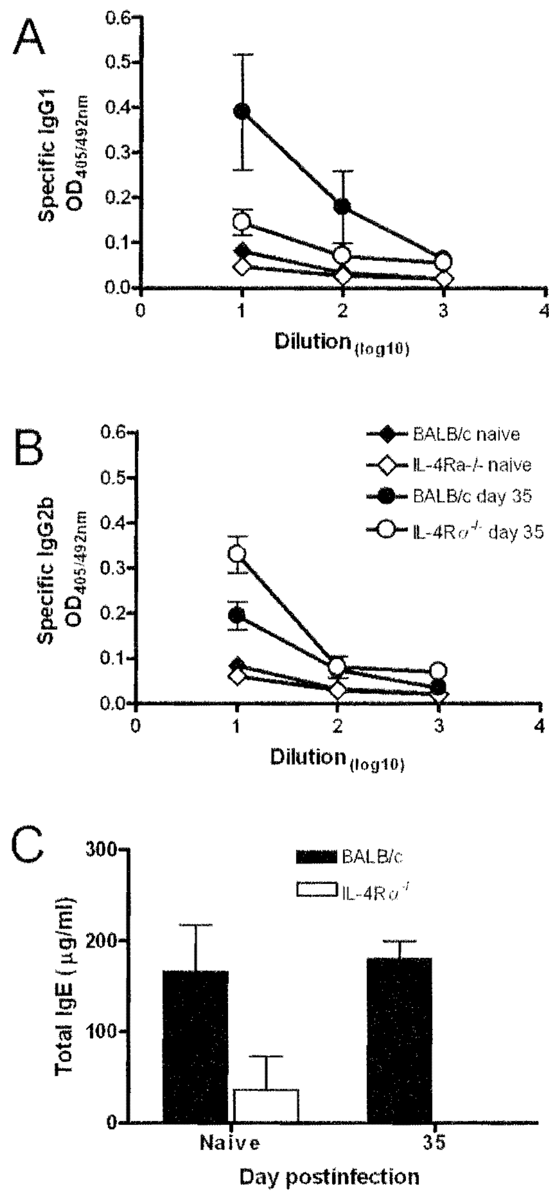


Figure 1.12. *S. obvelata*-specific IgG1 **(A)** and IgG2b **(B)** levels. Sera collected from infected and non-infected mice were analyzed by an end-point titration ELISA at day 0 and 35. Results are expressed as means of absorbance at 405/492nm. One representative of 3 independent experiments is shown. Total IgE kinetics **(C)** to mice infected with *S. obvelata*. Results are expressed as mean concentration (μg/ml). Four mice/group ± SD.

1.4.6. Eosinophilia, mastocytosis and goblet cell hyperplasia

To evaluate the effector mechanisms induced to expel *S. obvelata* we assayed Th2-assocaited eosinophilia, mastocytosis (mMCP-1) and goblet cell hyperplasia in a kinetic experiment. Blood and tissue samples from *S. obvelata* infected BALB/c and IL-4Rα$^{-/-}$ mice were analyzed for qualitative and quantitative responses. BALB/c mice showing complete clearance of worms by day 21 postinfection induced no significant eosinophilia, mastocytosis and goblet cell hyperplasia. In contrast, IL-4Rα$^{-/-}$ mice showed a significant increase in eosinophilia week 4 postinfection (Table 1.1), which correlated with the heavy worm burden observed at this time point (Fig. 1.9). Tissue mMCP-1 levels as well as goblet cell hyperplasia showed no induction throughout the infection (Table 1.1).

Table 1.1: Eosinophilia, mastocytosis and goblet cell hyperplasia in response to *Syphacia obvelata* infection

Analysis	Strain	Week					
		0	1	2	3	4	5
Peripheral blood eosinophilia[a]	BALB/c	200 ± 191	281 ± 109	169 ± 85.1	87.5 ± 87.8	244 ± 114	125 ± 45.6
	IL-4Rα$^{-/-}$	66.2 ± 59.2	100 ± 73.6	112 ± 77.7	125 ± 117	175 ± 73.6*	288 ± 111
EPO[b]	BALB/c	18.3 ± 5.55	11.9 ± 2.16	43.5 ± 40.7	12.3 ± 5.28	10.2 ± 1.54	13.5 ± 10.2
	IL-4Rα$^{-/-}$	13.4 ± 0.68	15.4 ± 2.11	12.8 ± 6.66	10.7 ± 2.94	10.1 ± 0.60*	16.5 ± 3.01
mMCP-1[b] (10^4)	BALB/c	7.40± 3.82	11.2 ± 4.29	14.6 ± 9.37	14.6 ± 7.40	20.5 ± 14.0	17.3 ± 8.19
	IL-4Rα$^{-/-}$	1.42 ± 0.08	0.92 ± 0.47	0.84 ± 2.72	0.60 ± 3.06	2.92 ± 1.30	8.05 ± 6.07
Goblet cell hyperplasia[c]	BALB/c	287 ± 73.7	199 ± 64.8	279 ± 115			
	IL-4Rα$^{-/-}$	210 ± 47.3	206 ± 49.3	230 ± 110			

[a] Peripheral blood eosinophilia was quantitated as the number of cells/ml of blood. Blood was collected from the tailvein and diluted (1:10) in Discombe's solution, which was prepared by mixing 1 volume of 1% aqueous eosin Y with 1 volume of acetone and 0.1M EDTA, and finally diluting 1:10 in water [219].

[b] Eosinophil peroxidase (EPO) and mouse mast cell protease-1 (mMCP-1) was assayed from the colon of *S. obvelata* infected mice

[c] PAS stained colon sections were examined under a light microscope for mucus producing goblet cells. The effector response was measured as the number of mucus producing goblet cells/2-4 colonic mucosa. Results are expressed as the means from independent experiments *, $p < 0.05$, significantly different from naïve. Four mice/group ± SD.

1.4.7. IL-13 signaling via IL-4Rα on macrophages, T cells and smooth muscle cells are not required for protection against *S. obvelata*

After identifying IL-13 as the dominant cytokine signaling through IL-4Rα, we investigated which cell types expressing this receptor are important in initiating expulsion against *S. obvelata*. Using cell-specific gene-targeted mice deficient for IL-4Rα, we assessed the worm burden of mice at day 35 postinfection. Globally deficient IL-4Rα mice were increasingly susceptible harboring high parasite loads to the BALB/c controls. All cell-specific IL-4Rα deficient mice elicited a resistant phenotype with a low worm recovery. Surprisingly mice devoid of IL-4Rα on smooth muscle cells showed complete clearance of pinworm in 35 days postinfection. These results showed that signaling of IL-4Rα on macrophages, T cells and smooth muscle cells (SMC) play no significance in the expulsion of the nematode.

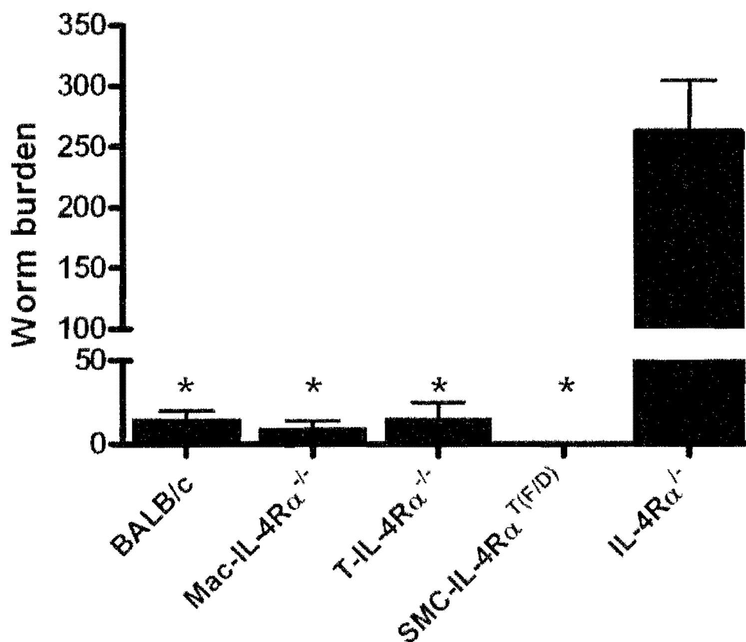


Figure 1.13. *S. obvelata* worm burden in cell-specific gene-targeted mice. Macrophage, T cells and smooth muscle cells deficient for IL-4Rα were inoculated orally with 500 pinworm eggs and analyzed at day 35 postinfection. *, $p < 0.05$, significantly different from globally deficient IL-4Rα mice. Three-four mice/group ± SD.

1.5 Discussion

Experimental murine models are important in providing an understanding of the factors contributing to resistance or susceptibility to diseases in both human and domesticated animals [222]. In order to understand host responses specific to a model disease it is imperative that experimental animals are kept in a pathogen free environment. The importance of sterility in housing and transporting laboratory animals is sometimes underestimated, which potentially increase the vulnerability of mice to adventitious pathogens [223]. In this study, we demonstrated the consequence of an outbreak in a transgenic mouse facility by a common and highly contagious mouse parasite, pinworm (*S. obvelata*). All mouse strains maintained within one of our SPF facilities were severely and chronically infected.

The degree of infection with *S. obvelata* was dependent on the mouse strains involved. Several inbred mouse strains (BALB/c and C57BL/6) and mice deficient for type 1 cytokines (IFN-γ$R^{-/-}$ and IL-12p$40^{-/-}$) were able to expel the worm and had low parasite burdens following infection. 129/Sv wild type mice showed complete resistance harboring no worms. Whether these mice rapidly expelled the GI nematode or completely inhibited its establishment within the cecum and colon would require further investigation. Nevertheless, mice deficient for the IL-4Rα, became severely and chronically infected, harboring approximately 1000 plus cecal dwelling worms. Resistance was mediated by IL-13 as mice deficient for the cytokine were highly susceptible and comparable to IL-4Rα deficient or IL-4/IL-13 double deficient mice. A similar IL-4-independent but IL-13-dependent role has been documented in *N. brasiliensis* infection [224,225] and highlights the important role of IL-13 in nematode infection.

In this study, all mice infected with *Syphacia obvelata*, including those naturally infected, consistently produced IFN-γ class switching isotype antibody, IgG2b. Detectable levels (A >0.2) above naïve controls were independent of mouse genetic background. Antibody production in transgenic knock-out mice infected with the GI nematode suggested that the increased IgG2b antibody production was IL-4-

dependent. Mice deficient of IL-12p40 provoked a more pronounced IgG2b antibody response (Fig. 1.8G) than that observed in IL-$4^{-/-}$ mice (Fig.1.8C and Fig. 1.12B). This however is highly unlikely as the cytokine IL-12 is known to activate macrophages to produce IFN-γ, responsible for IgM antibody class switching to IgG2a and IgG2b isotypes. In addition, an increase in IL-12-independent IgG2a was not observed and IgG2b antibody production was only shown in the one natural experiment. A more stringent analysis to conclude these findings would be required. It is important to note that pinworm egg antigens are not responsible for the IgG2b isotype antibody production, as gravid females migrate down the colon to deposit their eggs on the perianal region of their host. Deposition of eggs is triggered by the onset of air from the external environment [198].

Dai et al. (2001), studying *Echinococcus multiocularis* (alveolar echinococcosis) demonstrated that the major lectin-binding carbohydrate antigen of the helminth induces an IgG response independent of $\alpha\beta^+$ $CD4^+$ T cells [226]. The metacestode stage used in infectious studies consists of an inner, germinal layer representing the live parasite tissue and an outer, acellular laminated layer (LL) surrounding the entire metacestode. The LL has been suggested to protect metacestodes from the host immune response [227,228]. *Echinococcus multiocularis* infection induces both parasite-specific cell mediated and humoral immune responses [228]. Cellular immunity plays an important role in *E. multiocularis* protection and is characterized by the development of intrahepatic granulomas surrounding the parasite tissue [228]. Disease regression as well as progression, in both human patients and rodents correlates with a course-specific granuloma cell composition and induction of antigen-specific T-cell responses [229-231]. Antibody synthesis and isotype switching requires a cognate interaction between antigen-specific B cells and major histocompatibility complex class II (MHC II)-restricted $\alpha\beta^+CD4^+$ T cells [226]. However, there is evidence that B-cell activation and IgG antibody response can take place in the absence of T-cell help and do not require CD40-CD40L interactions [232,233]. Antigens that stimulate antibody production in the absence of MHC II-restricted T-cell help are classified as T-cell-independent (TI) antigens [232,234] and are divided into two groups namely; TI type 1 (TI-1) and TI-2 antigens. Lipopolysaccharide (LPS) is a TI-1 type antigen, which induces polyclonal activation of B cells. T cell-independent type 2 antigens such as

EM2 (G11) from *E. multiocularis* is not cognatively recognized in the context of MHC II restriction elements but are capable of stimulating antibody production in nude mice but not in *xid* mice. Peripheral B cells from *xid* mice are functionally impaired and are unresponsive to activation by mitogenic anti-Ig and TI-2 antigens [235,236]. TI-2 antigens are high molecular-weight polysaccharides containing multiple identical antigentic epitopes and demonstrate low *in vivo* degradability [226]. The tegument of *Syphacia obvelata* might well be composed of such carbohydrate molecules, in addition to excretory/secretory antigens which would explain the type 1 antibody response observed with IL-12p40$^{-/-}$ mice. An analysis of the constituents comprising the tegument surrounding *S. obvelata* could provide a more plausible explanation to the IgG isotype antibody response. It should be noted that the emerging interest in pattern recognition receptors of mammal cells have demonstrated that carbohydrate molecules coating GI nematodes play a vital role in their early detection by the innate immune response supporting our notion of such antigen within pinworm.

S. obvelata infection induced a transient Th2-type response, leading to a dominant type 2 accompanied antigen-specific B cell response, with high IgG1 antibodies. As expected from previous helminth infections, observed Th2 responses were IL-4Rα dependent with a shift towards Th1 responses (Fig. 1.11). Nevertheless, residual IL-4Rα-independent Th2 responses have also previously been published in other systems [237-240]. A striking observation was the absence of an increased polyclonal IgE antibody production during a primary *S. obvelata* infection. Polyclonal IgE synthesis is a hallmark response of nematode infections. Antigen-specific IgE responses were low in mice infected with 500 eggs. However, elevated polyclonal IgE production was demonstrated during continuous reinfection (Fig. 1.8D). The moderate response observed here, could be correlated to the low IL-4 production as IL-4 mediates IgE isotype switching [241]. In contrast, to *N. brasiliensis* where as little as 6 infective larvae are able to induce an IL-4 response in the draining MLN [242], *S. obvelata* induced only a very low response in the lymph node (unpublished data), despite infection with 500 eggs. Also, anti-CD3-restimulated spleen cells resulted in modest IL-4 production whilst IL-5 and IL-13 were strongly induced (Fig. 4C, D).

Immunomodulatory effects can broadly be divided in two overlapping subgroups: the induction of immunomodulatory cell types and the production of parasite-derived immunomodulatory molecules [54]. These mechanisms target key parts of the host immune system to avoid parasite destruction with minimal compromise to host survival. In our study, antigen-stimulated mesenteric lymph node cells draining the intestine where pinworm resides provoked a poor cytokine response. Both Th1 (IFN-γ) and Th2 (IL-4, IL-5 and IL-13) cytokine production was low in cells stimulate with parasite antigen and anti-CD3 T cell mitogen respectively. The Th immune response was more pronounced in spleen cells stimulated with anti-CD3 (Fig. 1.11).

The high concentration of *S. obvelata* antigen draining the mesenteric lymph node could explain the reduced cytokine production which upon reaching the spleen was diluted enough to induce a Th2 immune response. The concentration of pinworm antigen and whether this correlates with the reduced cytokine response in the lymph nodes would require further experimentation. To induce an effective adaptive immune response antigen processed by APC is needed to present the antigen to T cells in the context of MHC class II. Several steps in this process are mediated by cysteine proteases which are involved in the initial degradation of proteins in the endosomal-lysosomal compartment of the APC. Cysteine protease inhibitors or cystatins produced by nematodes are becoming more evident, interfering with the processing pathway [243]. Dainichi et al. demonstrated that a cystatin isolated from the GI nematode *N. brasiliensis* inhibits the processing of ovalbumin by lysosomal cysteine proteases [244]. A similar cysteine protease inhibitor has been identified in Haemonchus contortus [245]. Other protease inhibitors common to GI nematodes act to modulate the end stages of host immunological effector responses (mastocytosis), which we demonstrated was not induced in either *S. obvelata* resistant (BALB/c) or susceptible (IL-4Rα$^{-/-}$) mice (Table 1.1). In accordance with our *S. obvelata* experimental model, the potential of disarming potent chemical mediators is not known, but protease inhibitors have been characterized and an immunomodulatory role proposed in *Trichuris suis* [246], *Ascaris* [247,248] and *Ancylostoma ceylanicum* [249] nematode infections. Interestingly, we showed that antibodies directed against pinworm antigens cross-reacted with *N. brasiliensis* homogenates which suggest that the nematodes share molecules that are similar in structure. A homolog of the cysteine proteinase inhibitor

nippocystatin (NbCys) could possibly explain the immunomodulatory effects observed in our studies with *S. obvelata* infection. Parasite cystatins have been shown to have immunomodulatory effects. The downregulation of T-lymphocyte proliferation was shown in two independent studies with recombinant cystatin. Recombinant cystatin from the filarial parasite *Acanthocheilonema viteae* induce a non-specific suppressive effect [250] whereas recombinant NbCys showed only suppression of specific T cell proliferation [244]. Recombinant cystatin from *N. brasiliensis* similarly produce less Th1 and Th2 Ova-specific cytokines as observed with *S. obvelata* infected mice (Fig. 2.7) immunized to ovalbumin.

The three common effector mechanisms, eosinophilia, mastocytosis and goblet cell hyperplasia are characteristic features of infection with GI helminths. Their individual roles in the expulsion of nematodes have intensively been investigated. However, the precise nature of their role in worm expulsion remains to be defined. Studies showing a correlation between effector responses and parasite expulsion include *Strongyloides venezuelensis* and *Trichinella spiralis*, which are susceptible to the inhospitable environment created by mast cells. Similarly, eosinophils known for releasing potent cytotoxic and proinflammatory mediators have been demonstrated to protect against repeated exposure to GI parasites, such as *T. spiralis*. Goblet cell hyperplasia is associated with worm expulsion in *Strongyloides ratti* [171], *T. muris* [106] and *T. spiralis* [172,173] with *N. brasiliensis* [89,169,170] being the most efficient example, vulnerable to both the goblet cell hyperplactic response and a qualitative change in mucin glycoproteins [251]. In our study, mice infected with *S. obvelata* elicited no significant eosinophilia, mastocytosis and/or goblet cell hyperplasia suggesting that none of these effector mechanisms dominant at expelling the nematode. The isolated roles of these immunological effector mechanisms observed might be ineffective, however when acting together they may expel pinworm.

Although we had identified IL-13 signaling via IL-4Rα as the dominate pathway to pinworm resistance, the cells involved in the expulsion of the GI nematode remain to be determined. Cell-specific gene-targeted mice deficient for IL-4Rα on T cells effectively controlled a pinworm infection suggesting that IL-13 signaling via T cells is not required for expulsion. Murine T cells do not respond to stimulation by IL-13

[238] and as IL-4 and IL-13 share a common signaling pathway through the IL-4Rα chain, it is suffice to deduce that IL-4/IL-4Rα signaling on T cells play no role in *S. obvelata* expulsion. We have already shown that mice deficient of IL-4 cytokine production do not inhibit pinworm expulsion (Fig. 1.10) and that the cytokine possibly plays a role during chronic infection (Fig. 1.7). Previous studies have identified a role for alternatively activated macrophages in helminth infection and have demonstrated suppression of T cell proliferation [252] and downregulation of Th1 responses and immunopathology [34], respectively. To determine whether these macrophages are of any significance in our pinworm model, LysM^{cre}IL-4Rα$^{-/flox}$ mice (macrophage IL-4Rα$^{-/-}$ mice) were infected and their worm burden analyzed. Macrophage IL-4Rα$^{-/-}$ mice had no problem controlling the infection expelling a significant amount of worms to that observed in mice completely absent of IL-4Rα signaling (Fig. 1.13). Inflammation of the GI tract induced by nematode infections is accompanied by increased peristaltic movement of the gut and accelerated transit of its intestinal contents [162-164]. This muscle contractile and propulsive activity recently shown to be mediated by Th2 cytokines (IL-4 and IL-13) are believed to dislodge parasites already damaged by the effector immune response. In our study, no inhibition of pinworm expulsion was observed in smooth muscle IL-4Rα$^{-/-}$ mice to suggest that these cells elicit any response associated with worm clearance (Fig. 1.13). In addition, eosinophilia, mastocytosis and goblet cell hyperplasia was not induced in response to *S. obvelata* infection (Table 1.1).

Our findings are not surprising as it has been known for sometime that GI nematode expulsion is dependent on type 2 cytokines and that expulsion can occur in the absence of classical effector mechanisms associated with this type of response [15,106,253]. To unravel this mystery, many research groups have adopted global gene profiling and proteomic analyses of intestinal responses associated with worm expulsion in an attempt to identify novel type 2 cytokine-dependent immune effector mechanisms involved in immunity [254]. In three independent studies using either *T. muris* [254,255] or *T. spiralis* [256] infections, the authors identified up-regulation of a number of common epithelial-intrinsic genes which correlated with the period of worm expulsion. These included intelectins, a family of calcium-dependent galactose-binding lectins [257] resistin-like molecules-β, a small cysteine rich goblet cell specific

protein [258] and machinery associated with ion exchange and barrier function [259-261]. Pinworm infections are asymptomatic and few if any have been associated with inflammation of the GI tract. The pathogens have evolved well orchestrated evasive/suppressive mechanisms and possibly exert minimal parasite-host interaction to initiate a robust immune response. Whether these novel molecules and pathways associated with the epithelium of the intestine, the niche of *S. obvelata*, play any role in the IL-13-mediated worm expulsion is unknown and would required further investigation.

In conclusion, we have shown that *S. obvelata* induce a Th2-type immune response with IL-13 the dominate cytokine required for protection.

1.6 References

1. Roitt I, Brostoff J, Male D: Introduction to Immunology. Immunology. Edited by Crowe L, Furn R. Edinburgh, Mosby International Limited, 1998, pp 1-11
2. Prescott L, Harley JP, Klein DA: Medical Microbiology. USA, Wm. C. Brown Publishers, 1996
3. Playfair JHL: Immunology at a glance. London, Blackwell Scientific Publications, 1992
4. Raven PH, Johnson GB: Biology. Dubuque, Wm. C. Brown Publishers, 1996
5. Janeway CAJ, Travers P: The development of B lymphocytes. ImmunoBiology: The immune system in health and disease. Edited by 3rd. London and New York, Current Biology and Garland Publishing Inc., 1997
6. Male D: Introduction to the immune system. Immunology. Edited by Crowe L, Furn R. Edinburgh, Mosby, 2001, pp 1-12
7. Mygind N, Dahl R, Pedersen S, Thestrup-Pedersen K: Essential Allergy, Blackwell Science, 1996
8. Lydyard PM, Grossi CE: Cells, tissue and organs of the immune system. Immunology. Edited by Crowe L, Furn R. Edinburgh, Mosby, 2001
9. Roitt IM: The synthesis of antibody. Essential Immunology. Oxford, 1977, pp 47-100
10. Janeway CAJ, Travers P: Host defense against infection. Immunology: The Immune System in Health and Disease. Edited by Austin P, Lawrence E. New York, Garland Publishing Inc., 1997, pp 9.7-9.52
11. Williams AF: A year in the life of the immunology superfamily. Immunology Today 1987, 8:298-303

12. www.tmd.ac.jp/artsci/biol/textlife/immunology.htm.Toyko:

13. Sher A, Gazzinelli RT, Oswald IP, Clerici M, Kullberg M, Pearce EJ, Berzofsky JA, Mosmann TR, James SL, Morse HC, 3rd: Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. Immunol Rev 1992, 127:183-204

14. Sher A, Coffman RL: Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu Rev Immunol 1992, 10:385-409

15. Betts CJ, Else KJ: Mast cells, eosinophils and antibody-mediated cellular cytotoxicity are not critical in resistance to Trichuris muris. Parasite Immunol 1999, 21:45-52

16. Fiorentino DF, Bond MW, Mosmann TR: Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 1989, 170:2081-2095

17. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffinan RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986, 136:2348-2357

18. Mosmann TR, Coffman RL: Heterogeneity of cytokine secretion patterns and functions of helper T cells. Adv Immunol 1989, 46:111-147

19. Cherwinski HM, Schumacher JH, Brown KD, Mosmann TR: Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J Exp Med 1987, 166:1229-1244

20. Stvritinova V, Jakubovsky J, Hulin I: Inflammation and Fever. Pathophysiology - Principles of Disease. Slovakia, Computing Centre, Slovak Academy of Sciences, 1995
21. Mota I: Acivity of immune cells. Fundamentals of Immunology. New York, Berlin, Heidelberg, Springer-Verlag, 1986, pp 59-61
22. Noel W, Raes G, Hassanzadeh Ghassabeh G, De Baetselier P, Beschin A: Alternatively activated macrophages during parasite infections. Trends Parasitol 2004, 20:126-133
23. Gordon S: Alternative activation of macrophages. Nat Rev Immunol 2003, 3:23-35
24. Loke P, Nair MG, Parkinson J, Guiliano D, Blaxter M, Allen JE: IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. BMC Immunol 2002, 3:7
25. Raes G, De Baetselier P, Noel W, Beschin A, Brombacher F, Hassanzadeh Gh G: Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. J Leukoc Biol 2002, 71:597-602
26. Munder M, Eichmann K, Modolell M: Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. J Immunol 1998, 160:5347-5354
27. Goerdt S, Orfanos CE: Other functions, other genes: alternative activation of antigen-presenting cells. Immunity 1999, 10:137-142

28. Bonecchi R, Sozzani S, Stine JT, Luini W, D'Amico G, Allavena P, Chantry D, Mantovani A: Divergent effects of interleukin-4 and interferon-gamma on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. Blood 1998, 92:2668-2671

29. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O: Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999, 11:81-88

30. Schebesch C, Kodelja V, Muller C, Hakij N, Bisson S, Orfanos CE, Goerdt S: Alternatively activated macrophages actively inhibit proliferation of peripheral blood lymphocytes and CD4+ T cells in vitro. Immunology 1997, 92:478-486

31. Namangala B, De Baetselier P, Noel W, Brys L, Beschin A: Alternative versus classical macrophage activation during experimental African trypanosomosis. J Leukoc Biol 2001, 69:387-396

32. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A: IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991, 147:3815-3822

33. Hegele RG: The pathology of asthma: brief review. Immunopharmacology 2000, 48:257-262

34. Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, Leeto M, Kirsch R, Hall P, Mossmann H, Claussen B, Forster I, Brombacher F: Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. Immunity 2004, 20:623-635

35. Urban JF, Jr., Noben-Trauth N, Schopf L, Madden KB, Finkelman FD: Cutting edge: IL-4 receptor expression by non-bone marrow-derived cells is required to expel gastrointestinal nematode parasites. J Immunol 2001, 167:6078-6081

36. McKenzie GJ, Bancroft A, Grencis RK, McKenzie AN: A distinct role for interleukin-13 in Th2-cell-mediated immune responses. Curr Biol 1998, 8:339-342

37. Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE: Helminth parasites--masters of regulation. Immunol Rev 2004, 201:89-116

38. Male D: Cell-mediated cytotoxicty. Immunology. Edited by Crowe L, Furn R. Edinburgh, Mosby, 2001, pp 164-170

39. Mohrs M, Ledermann B, Kohler G, Dorfmuller A, Gessner A, Brombacher F: Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. J Immunol 1999, 162:7302-7308

40. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G: Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 1993, 362:245-248

41. Janeway CAJ, Travers P: T-cell mediated immunity. Immunology: The immune system in health and disease. Edited by 2nd, Current Biology Ltd and Garland Publishing, 1996, pp 7:25-27:27

42. Decker JM: Immunology: Welcome to immunology 419, 2006

43. Kunkel SL, Chensue SW, Strieter RM: Chemokines and the Inflammatory Response. Cytokines in Health and Disease. Edited by Remick DG, Friedland JS. New York, Marcelm Dekker, 1997, pp 121-131

44. Male D: Cell migration and inflammation. Immunology. Edited by Crowe L, Furn R. Edinburgh, Mosby, 2001, pp 47-64

45. Balkwill F: Cytokines and cytokine receptors. Immunology. Edited by Roitt I, Brostoff J, Male D. Spain, Mosby, 2001, pp 119-123

46. Shepherd VL, Abdolrasulnia R: Cytokine Receptors. Cytokines in Health and Disease. Edited by Remick DG, Friedland JS. New York, Marcelm Dekker, 1997, pp 263-279

47. Harvath L, Falk W, Leonard EJ: Rapid quantitation of neutrophil chemotaxis: use of a polyvinylpyrrolidone-free polycarbonate membrane in a multiwell assembly. J Immunol Methods 1980, 37:39-45

48. Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV: Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 1984, 312:724-729

49. Remick DG, Scales WE, Chensue SW, Kunkel SL: The pethophysiology of interlekins and tumor necrosis factors. Cellular Pathophysiology. Edited by Sayeed M. Boca Raton, Florida, USA, CRC Press, 1989, pp 41-89

50. Markell EK, Voge M, John AT: Chapter 2: Parasites, Parasitism, and Host Relations. Medical Parasitology. Edited by Ozmat S. Philadelphia, W. B. Saunders Company, 1992, pp 5-14

51. Murray PR, Kobayashi GS, Pfaller MA, Rosenthal KS: Intestinal and Urogenital Protozoa. Medical Microbiology. Edited by Farrell R. St. Louis, Missouri, Mosby, 1994, pp 448-462

52. Murray PR, Kobayashi GS, Pfaller MA, Rosenthal KS: Blood and Tissue Protozoa. Medical Microbiology. Edited by Farrell R. St. Louis, Missouri, Mosby, 1994, pp 463-480

53. Murray PR, Kobayashi GS, Pfaller MA, Rosenthal KS: Trematodes. Medical Microbiology. Edited by Farrell R. St. Louis, Missouri, Mosby, 1994, pp 500-510

54. Myers P: Arthropoda (On-Line), Animal Diversity Web, 2001

55. Goddard J: Arthropods, Tongue worms, Leeches, and Arthopod-borne Diseases. Tropical Infectious Disease: Principes, Pathogens, and Pratice. Edited by Guerrant RL, Walker DH, Weller PF. Philadelphia, Elsevier Churchill Livingstone, 2006, pp 1370-1385

56. Barbour AG: Relapsing Fever and Other Borrelia Disease. Tropical Infectious Disease: Principles, Pathogens, and Practice. Edited by Guerrant RL, Walker DH, Weller PF. Philadelphia, Elsevier, Churchill Livingston, 2006, pp 499-510

57. Murray PR, Kobayashi GS, Pfaller MA, Rosenthal KS: Parasitic Classification and Physiology. Medical Microbiology. Edited by Farrell R. St. Louis, Missouri, Mosby, 1994, pp 51-57

58. Jeronimo SMB, Q SA, Perason RD: Leishmaniasis. Tropical Infectious Disease: Principles, Pathogens, and Practice. Edited by Guerrant RL, Walker DH, Weller PF. Philadelphia, Elsevier, Churchill Livingston, 2006, pp 1095-1113

59. Pepin J, Donelson JE: African Trypanosomiasis (Sleeping Sickness). Tropical Infectious Disease: Principles, Pathogens, and Practice. Edited by Guerrant RL, Walker DH, Weller PF. Philadelphia, Elsevier, Churchill Livingston, 2006, pp 1072-1081

60. http://www.who.int/wormcontrol/en/:

61. Crompton DW: How much human helminthiasis is there in the world? J Parasitol 1999, 85:397-403

62. Stepek G, Buttle DJ, Duce IR, Behnke JM: Human gastrointestinal nematode infections: are new control methods required? Int J Exp Pathol 2006, 87:325-341

63. Booth M, Bundy DA, Albonico M, Chwaya HM, Alawi KS, Savioli L: Associations among multiple geohelminth species infections in schoolchildren from Pemba Island. Parasitology 1998, 116 (Pt 1):85-93

64. Clinch CR, Stephens MB: Case description of ascariasis. Arch Fam Med 2000, 9:1193-1194

65. Gilles HM: Gastrointestinal helminthiasis. Br Med J 1968, 2:475-477

66. Gonzalez S, De la Cabada FJ: Parasitic infections of the colon and rectum. Baillieres Clin Gastroenterol 1987, 1:447-467

67. Kucik CJ, Martin GL, Sortor BV: Common intestinal parasites. Am Fam Physician 2004, 69:1161-1168

68. Gilgen DD, Mascie-Taylor CG, Rosetta LL: Intestinal helminth infections, anaemia and labour productivity of female tea pluckers in Bangladesh. Trop Med Int Health 2001, 6:449-457

69. Crompton DW: Nutritional aspects of infection. Trans R Soc Trop Med Hyg 1986, 80:697-705

70. Albonico M, Stoltzfus RJ, Savioli L, Tielsch JM, Chwaya HM, Ercole E, Cancrini G: Epidemiological evidence for a differential effect of hookworm species, Ancylostoma duodenale or Necator americanus, on iron status of children. Int J Epidemiol 1998, 27:530-537

71. Bundy DA, Chan MS, Savioli L: Hookworm infection in pregnancy. Trans R Soc Trop Med Hyg 1995, 89:521-522

72. Coop RL, Kyriazakis I: Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. Trends Parasitol 2001, 17:325-330

73. Stephenson LS, Latham MC, Ottesen EA: Malnutrition and parasitic helminth infections. Parasitology 2000, 121 Suppl:S23-38

74. Dossa RA, Ategbo EA, de Koning FL, van Raaij JM, Hautvast JG: Impact of iron supplementation and deworming on growth performance in preschool Beninese children. Eur J Clin Nutr 2001, 55:223-228

75. Stephensen CB: Burden of infection on growth failure. J Nutr 1999, 129:534S-538S

76. Bentwich Z, Kalinkovich A, Weisman Z, Borkow G, Beyers N, Beyers AD: Can eradication of helminthic infections change the face of AIDS and tuberculosis? Immunol Today 1999, 20:485-487

77. Elliott AM, Kyosiimire J, Quigley MA, Nakiyingi J, Watera C, Brown M, Joseph S, French N, Gilks CF, Whitworth JA: Eosinophilia and progression to active tuberculosis in HIV-1-infected Ugandans. Trans R Soc Trop Med Hyg 2003, 97:477-480

78. Othieno C, Hirsch CS, Hamilton BD, Wilkinson K, Ellner JJ, Toossi Z: Interaction of Mycobacterium tuberculosis-induced transforming growth factor beta1 and interleukin-10. Infect Immun 1999, 67:5730-5735

79. Bentwich Z, Kalinkovich A, Weisman Z: Immune activation is a dominant factor in the pathogenesis of African AIDS. Immunol Today 1995, 16:187-191

80. Borkow G, Bentwich Z: Eradication of helminthic infections may be essential for successful vaccination against HIV and tuberculosis. Bull World Health Organ 2000, 78:1368-1369

81. Pinder J, Fowler R, Bannister L, Dluzewski A, Mitchell GH: Motile systems in malaria merozoites: how is the red blood cell invaded? Parasitol Today 2000, 16:240-245

82. Bradley JE: Immunity to protozoa and worms. Immunology. Edited by Crowe L, Furn R. Edinburgh, Mosby, 2001, pp 259-276

83. Mulcahy G, O'Neill S, Donnelly S, Dalton JP: Helminths at mucosal barriers--interaction with the immune system. Adv Drug Deliv Rev 2004, 56:853-868

84. Janeway CAJ, Medzhitov R: Innate immune recognition. Ann Rev Immunol 2002, 20:853-868

85. Medzhitov R, Janeway CA, Jr.: Decoding the patterns of self and nonself by the innate immune system. Science 2002, 296:298-300

86. Moncada DM, Kammanadiminti SJ, Chadee K: Mucin and Toll-like receptors in host defense against intestinal parasites. Trends Parasitol 2003, 19:305-311

87. Juntunen M, Kirjavainen PV, Ouwehand AC, Salminen SJ, Isolauri E: Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection. Clin Diagn Lab Immunol 2001, 8:293-296

88. Specian RD, Oliver MG: Functional biology of intestinal goblet cells. Am J Physiol 1991, 260:C183-193

89. Miller HR: Gastrointestinal mucus, a medium for survival and for elimination of parasitic nematodes and protozoa. Parasitology 1987, 94 Suppl:S77-100

90. Allen A: Physiology of Gastrointestinal Tract. Edited by Johnson LR. New York, Raven Press, 1981, pp 617-639

91. Karlsson NG, Olson FJ, Jovall PA, Andersch Y, Enerback L, Hansson GC: Identification of transient glycosylation alterations of sialylated mucin oligosaccharides during infection by the rat intestinal parasite Nippostrongylus brasiliensis. Biochem J 2000, 350 Pt 3:805-814

92. Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, Knight PA, Donaldson DD, Lazar MA, Miller HR, Schad GA, Scott P, Wu GD: RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. Proc Natl Acad Sci U S A 2004, 101:13596-13600

93. Klabunde J, Berger J, Jensenius JC, Klinkert MQ, Zelck UE, Kremsner PG, Kun JF: Schistosoma mansoni: adhesion of mannan-binding lectin to surface glycoproteins of cercariae and adult worms. Exp Parasitol 2000, 95:231-239

94. Hong Y, Kim CW, Ghebrehiwet B: Trichinella spiralis: activation of complement by infective larvae, adults, and newborn larvae. Exp Parasitol 1992, 74:290-299

95. Santoro F, Ouaissi MA, Pestel J, Capron A: Interaction between Schistosoma mansoni and the complement system: binding of C1q to schistosomula. J Immunol 1980, 124:2886-2891

96. Haslam SM, Houston KM, Harnett W, Reason AJ, Morris HR, Dell A: Structural studies of N-glycans of filarial parasites. Conservation of phosphorylcholine-substituted glycans among species and discovery of novel chito-oligomers. J Biol Chem 1999, 274:20953-20960

97. Bout D, Joseph M, Pontet M, Vorng H, Deslee D, Capron A: Rat resistance to schistosomiasis: platelet-mediated cytotoxicity induced by C-reactive protein. Science 1986, 231:153-156

98. Appelmelk BJ, van Die I, van Vliet SJ, Vandenbroucke-Grauls CM, Geijtenbeek TB, van Kooyk Y: Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. J Immunol 2003, 170:1635-1639

99. McGuinness DH, Dehal PK, Pleass RJ: Pattern recognition molecules and innate immunity to parasites. Trends Parasitol 2003, 19:312-319

100. Geijtenbeek TB, Van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, Appelmelk B, Van Kooyk Y: Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med 2003, 197:7-17

101. Xu X, Remold HG, Caulfield JP: Potential role for scavenger receptors of human monocytes in the killing of Schistosoma mansoni. Am J Pathol 1993, 142:685-689

102. Ehlers MR: CR3: a general purpose adhesion-recognition receptor essential for innate immunity. Microbes Infect 2000, 2:289-294

103. Mastellos D, Lambris JD: Complement: more than a 'guard' against invading pathogens? Trends Immunol 2002, 23:485-491

104. Akira S, Takeda K, Kaisho T: Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001, 2:675-680

105. van der Kleij D, Latz E, Brouwers JF, Kruize YC, Schmitz M, Kurt-Jones EA, Espevik T, de Jong EC, Kapsenberg ML, Golenbock DT, Tielens AG, Yazdanbakhsh M: A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. J Biol Chem 2002, 277:48122-48129

106. Else KJ, Finkelman FD: Intestinal nematode parasites, cytokines and effector mechanisms. Int J Parasitol 1998, 28:1145-1158

107. Grencis RK: Th2-mediated host protective immunity to intestinal nematode infections. Philos Trans R Soc Lond B Biol Sci 1997, 352:1377-1384

108. Bancroft AJ, McKenzie AN, Grencis RK: A critical role for IL-13 in resistance to intestinal nematode infection. J Immunol 1998, 160:3453-3461

109. Else KJ, Finkelman FD, Maliszewski CR, Grencis RK: Cytokine-mediated regulation of chronic intestinal helminth infection. J Exp Med 1994, 179:347-351

110. Ferlin WG, Severinson E, Strom L, Heath AW, Coffman RL, Ferrick DA, Howard MC: CD40 signaling induces interleukin-4-independent IgE switching in vivo. Eur J Immunol 1996, 26:2911-2915

111. Urban JF, Jr., Noben-Trauth N, Donaldson DD, Madden KB, Morris SC, Collins M, Finkelman FD: IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. Immunity 1998, 8:255-264

112. Urban JF, Jr., Schopf L, Morris SC, Orekhova T, Madden KB, Betts CJ, Gamble HR, Byrd C, Donaldson D, Else K, Finkelman FD: Stat6 signaling promotes protective immunity against Trichinella spiralis through a mast cell- and T cell-dependent mechanism. J Immunol 2000, 164:2046-2052

113. Urban JF, Jr., Katona IM, Paul WE, Finkelman FD: Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. Proc Natl Acad Sci U S A 1991, 88:5513-5517

114. Zurawski SM, Chomarat P, Djossou O, Bidaud C, McKenzie AN, Miossec P, Banchereau J, Zurawski G: The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin-13 receptor. J Biol Chem 1995, 270:13869-13878

115. de Vries JE: The role of IL-13 and its receptor in allergy and inflammatory responses. J Allergy Clin Immunol 1998, 102:165-169

116. Smerz-Bertling C, Duschl A: Both interleukin 4 and interleukin 13 induce tyrosine phosphorylation of the 140-kDa subunit of the interleukin 4 receptor. J Biol Chem 1995, 270:966-970

117. Yu CR, Kirken RA, Malabarba MG, Young HA, Ortaldo JR: Differential regulation of the Janus kinase-STAT pathway and biologic function of IL-13 in primary human NK and T cells: a comparative study with IL-4. J Immunol 1998, 161:218-227

118. Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, Friedmann MC, Miyajima A, Puri RK, Paul WE, et al.: Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. Science 1993, 262:1880-1883

119. Wang LM, Keegan A, Frankel M, Paul WE, Pierce JH: Signal transduction through the IL-4 and insulin receptor families. Stem Cells 1995, 13:360-368

120. Donaldson DD, Whitters MJ, Fitz LJ, Neben TY, Finnerty H, Henderson SL, O'Hara RM, Jr., Beier DR, Turner KJ, Wood CR, Collins M: The murine IL-13 receptor alpha 2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha 1. J Immunol 1998, 161:2317-2324

121. Takeda K, Kamanaka M, Tanaka T, Kishimoto T, Akira S: Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. J Immunol 1996, 157:3220-3222

122. Palmer-Crocker RL, Hughes CC, Pober JS: IL-4 and IL-13 activate the JAK2 tyrosine kinase and Stat6 in cultured human vascular endothelial cells through a common pathway that does not involve the gamma c chain. J Clin Invest 1996, 98:604-609

123. Schnyder B, Lugli S, Feng N, Etter H, Lutz RA, Ryffel B, Sugamura K, Wunderli-Allenspach H, Moser R: Interleukin-4 (IL-4) and IL-13 bind to a shared heterodimeric complex on endothelial cells mediating vascular cell adhesion molecule-1 induction in the absence of the common gamma chain. Blood 1996, 87:4286-4295

124. Murata T, Noguchi PD, Puri RK: IL-13 induces phosphorylation and activation of JAK2 Janus kinase in human colon carcinoma cell lines: similarities between IL-4 and IL-13 signaling. J Immunol 1996, 156:2972-2978

125. Keegan AD, Johnston JA, Tortolani PJ, McReynolds LJ, Kinzer C, O'Shea JJ, Paul WE: Similarities and differences in signal transduction by interleukin 4 and interleukin 13: analysis of Janus kinase activation. Proc Natl Acad Sci U S A 1995, 92:7681-7685

126. Obiri NI, Debinski W, Leonard WJ, Puri RK: Receptor for interleukin 13. Interaction with interleukin 4 by a mechanism that does not involve the common gamma chain shared by receptors for interleukins 2, 4, 7, 9, and 15. J Biol Chem 1995, 270:8797-8804

127. He YW, Malek TR: The IL-2 receptor gamma c chain does not function as a subunit shared by the IL-4 and IL-13 receptors. Implication for the structure of the IL-4 receptor. J Immunol 1995, 155:9-12

128. Welham MJ, Learmonth L, Bone H, Schrader JW: Interleukin-13 signal transduction in lymphohemopoietic cells. Similarities and differences in signal transduction with interleukin-4 and insulin. J Biol Chem 1995, 270:12286-12296

129. Hilton DJ, Zhang JG, Metcalf D, Alexander WS, Nicola NA, Willson TA: Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. Proc Natl Acad Sci U S A 1996, 93:497-501

130. Zhang JG, Hilton DJ, Willson TA, McFarlane C, Roberts BA, Moritz RL, Simpson RJ, Alexander WS, Metcalf D, Nicola NA: Identification, purification, and characterization of a soluble interleukin (IL)-13-binding protein. Evidence that it is distinct from the cloned Il-13 receptor and Il-4 receptor alpha-chains. J Biol Chem 1997, 272:9474-9480

131. Aman MJ, Tayebi N, Obiri NI, Puri RK, Modi WS, Leonard WJ: cDNA cloning and characterization of the human interleukin 13 receptor alpha chain. J Biol Chem 1996, 271:29265-29270

132. Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N, Ferrara P: Cloning of the human IL-13R alpha1 chain and reconstitution with the IL4R alpha of a functional IL-4/IL-13 receptor complex. FEBS Lett 1997, 401:163-166

133. Gauchat JF, Schlagenhauf E, Feng NP, Moser R, Yamage M, Jeannin P, Alouani S, Elson G, Notarangelo LD, Wells T, Eugster HP, Bonnefoy JY: A novel 4-kb interleukin-13 receptor alpha mRNA expressed in human B, T, and endothelial cells encoding an alternate type-II interleukin-4/interleukin-13 receptor. Eur J Immunol 1997, 27:971-978

134. Feng N, Lugli SM, Schnyder B, Gauchat JF, Graber P, Schlagenhauf E, Schnarr B, Wiederkehr-Adam M, Duschl A, Heim MH, Lutz RA, Moser R: The interleukin-4/interleukin-13 receptor of human synovial fibroblasts: overexpression of the nonsignaling interleukin-13 receptor alpha2. Lab Invest 1998, 78:591-602

135. Ruetten H, Thiemermann C: Interleukin-13 is a more potent inhibitor of the expression of inducible nitric oxide synthase in smooth muscle cells than in macrophages: a comparison with interleukin-4 and interleukin-10. Shock 1997, 8:409-414

136. Brombacher F: The role of interleukin-13 in infectious diseases and allergy. Bioessays 2000, 22:646-656

137. Zurawski SM, Vega F, Jr., Huyghe B, Zurawski G: Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. Embo J 1993, 12:2663-2670

138. Li L, Xia Y, Nguyen A, Lai YH, Feng L, Mosmann TR, Lo D: Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. J Immunol 1999, 162:2477-2487

139. Endo T, Ogushi F, Sone S: LPS-dependent cyclooxygenase-2 induction in human monocytes is down-regulated by IL-13, but not by IFN-gamma. J Immunol 1996, 156:2240-2246

140. Doherty TM, Kastelein R, Menon S, Andrade S, Coffman RL: Modulation of murine macrophage function by IL-13. J Immunol 1993, 151:7151-7160

141. Sozzani P, Cambon C, Vita N, Seguelas MH, Caput D, Ferrara P, Pipy B: Interleukin-13 inhibits protein kinase C-triggered respiratory burst in human monocytes. Role of calcium and cyclic AMP. J Biol Chem 1995, 270:5084-5088

142. de Waal Malefyt R, Figdor CG, de Vries JE: Effects of interleukin 4 on monocyte functions: comparison to interleukin 13. Res Immunol 1993, 144:629-633

143. Yanagawa H, Sone S, Haku T, Mizuno K, Yano S, Ohmoto Y, Ogura T: Contrasting effects of interleukin-13 on interleukin-1 receptor antagoist and proinflammatory cytokine production by human alveolar macrophages. Am J Respir Cell Mol Biol 1995, 12:71-76

144. Colotta F, Saccani S, Giri JG, Dower SK, Sims JE, Introna M, Mantovani A: Regulated expression and release of the IL-1 decoy receptor in human mononuclear phagocytes. J Immunol 1996, 156:2534-2541

145. Dubois GR, Schweizer RC, Versluis C, Bruijnzeel-Koomen CA, Bruijnzeel PL: Human eosinophils constitutively express a functional interleukin-4 receptor: interleukin-4 -induced priming of chemotactic responses and induction of PI-3 kinase activity. Am J Respir Cell Mol Biol 1998, 19:691-699

146. Barner M, Mohrs M, Brombacher F, Kopf M: Differences between IL-4R alpha-deficient and IL-4-deficient mice reveal a role for IL-13 in the regulation of Th2 responses. Curr Biol 1998, 8:669-672

147. Urban JF, Jr., Maliszewski CR, Madden KB, Katona IM, Finkelman FD: IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice. J Immunol 1995, 154:4675-4684

148. Collins SM: The immunomodulation of enteric neuromuscular function: implications for motility and inflammatory disorders. Gastroenterology 1996, 111:1683-1699

149. Behm CA, Ovington KS: The role of eosinophils in parasitic helminth infections: insights from genetically modified mice. Parasitol Today 2000, 16:202-209

150. Kopf M, Brombacher F, Hodgkin PD, Ramsay AJ, Milbourne EA, Dai WJ, Ovington KS, Behm CA, Kohler G, Young IG, Matthaei KI: IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. Immunity 1996, 4:15-24

151. Herndon FJ, Kayes SG: Depletion of eosinophils by anti-IL-5 monoclonal antibody treatment of mice infected with Trichinella spiralis does not alter parasite burden or immunologic resistance to reinfection. J Immunol 1992, 149:3642-3647

152. Coffman RL, Seymour BW, Hudak S, Jackson J, Rennick D: Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. Science 1989, 245:308-310

153. Vallance BA, Matthaei KI, Sanovic S, Young IG, Collins SM: Interleukin-5 deficient mice exhibit impaired host defence against challenge Trichinella spiralis infections. Parasite Immunol 2000, 22:487-492

154. Sturrock RF, Kimani R, Cottrell BJ, Butterworth AE, Seitz HM, Siongok TK, Houba V: Observations on possible immunity to reinfection among Kenyan schoolchildren after treatment for Schistosoma mansoni. Trans R Soc Trop Med Hyg 1983, 77:363-371

155. Hagan P, Wilkins HA, Blumenthal UJ, Hayes RJ, Greenwood BM: Eosinophilia and resistance to Schistosoma haematobium in man. Parasite Immunol 1985, 7:625-632

156. Befus AD, Bienenstock J: Immunologically mediated intestinal mastocytosis in Nippostrongylus brasiliensis-infected rats. Immunology 1979, 38:95-101

157. Abe T, Nawa Y: Worm expulsion and mucosal mast cell response induced by repetitive IL-3 administration in Strongyloides ratti-infected nude mice. Immunology 1988, 63:181-185

158. Donaldson LE, Schmitt E, Huntley JF, Newlands GF, Grencis RK: A critical role for stem cell factor and c-kit in host protective immunity to an intestinal helminth. Int Immunol 1996, 8:559-567

159. Khan AI, Horii Y, Tiuria R, Sato Y, Nawa Y: Mucosal mast cells and the expulsive mechanisms of mice against Strongyloides venezuelensis. Int J Parasitol 1993, 23:551-555

160. Lantz CS, Boesiger J, Song CH, Mach N, Kobayashi T, Mulligan RC, Nawa Y, Dranoff G, Galli SJ: Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. Nature 1998, 392:90-93

161. Aoki I, Itoh S, Yokota S, Tanaka S, Ishii N, Okuda K, Minami M, Klinman DM: Contribution of mast cells to the T helper 2 response induced by simultaneous subcutaneous and oral immunization. Immunology 1999, 98:519-524

162. Castro GA, Badial-Aceves F, Smith JW, Dudrick SJ, Weisbrodt NW: Altered small bowel propulsion associated with parasitism. Gastroenterology 1976, 71:620-625

163. Vallance BA, Blennerhassett PA, Collins SM: Increased intestinal muscle contractility and worm expulsion in nematode-infected mice. Am J Physiol 1997, 272:G321-327

164. Vermillion DL, Collins SM: Increased responsiveness of jejunal longitudinal muscle in Trichinella-infected rats. Am J Physiol 1988, 254:G124-129

165. Akiho H, Blennerhassett P, Deng Y, Collins SM: Role of IL-4, IL-13, and STAT6 in inflammation-induced hypercontractility of murine smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 2002, 282:G226-232

166. Zhao A, McDermott J, Urban JF, Jr., Gause W, Madden KB, Yeung KA, Morris SC, Finkelman FD, Shea-Donohue T: Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. J Immunol 2003, 171:948-954

167. Khan WI, Richard M, Akiho H, Blennerhasset PA, Humphreys NE, Grencis RK, Van Snick J, Collins SM: Modulation of intestinal muscle contraction by interleukin-9 (IL-9) or IL-9 neutralization: correlation with worm expulsion in murine nematode infections. Infect Immun 2003, 71:2430-2438

168. Vallance BA, Collins SM: The effect of nematode infection upon intestinal smooth muscle function. Parasite Immunol 1998, 20:249-253

169. Miller HR, Nawa Y: Nippostrongylus brasiliensis: intestinal goblet-cell response in adoptively immunized rats. Exp Parasitol 1979, 47:81-90

170. Khan WI, Abe T, Ishikawa N, Nawa Y, Yoshimura K: Reduced amount of intestinal mucus by treatment with anti-CD4 antibody interferes with the spontaneous cure of Nippostrongylus brasiliensis-infection in mice. Parasite Immunol 1995, 17:485-491

171. Carroll SM, Mayrhofer G, Dawkins HJ, Grove DI: Kinetics of intestinal lamina propria mast cells, globule leucocytes, intraepithelial lymphocytes, goblet cells and eosinophils in murine strongyloidiasis. Int Arch Allergy Appl Immunol 1984, 74:311-317

172. Garside P, Grencis RK, Mowat AM: T lymphocyte dependent enteropathy in murine Trichinella spiralis infection. Parasite Immunol 1992, 14:217-225

173. Ishikawa N, Wakelin D, Mahida YR: Role of T helper 2 cells in intestinal goblet cell hyperplasia in mice infected with Trichinella spiralis. Gastroenterology 1997, 113:542-549

174. Nawa Y, Ishikawa N, Tsuchiya K, Horii Y, Abe T, Khan AI, Bing S, Itoh H, Ide H, Uchiyama F: Selective effector mechanisms for the expulsion of intestinal helminths. Parasite Immunol 1994, 16:333-338

175. Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK: Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. Science 2005, 308:1463-1465

176. Potten CS: Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philos Trans R Soc Lond B Biol Sci 1998, 353:821-830

177. Pritchard DI, Williams DJ, Behnke JM, Lee TD: The role of IgG1 hypergammaglobulinaemia in immunity to the gastrointestinal nematode Nematospiroides dubius. The immunochemical purification, antigen-specificity and in vivo anti-parasite effect of IgG1 from immune serum. Immunology 1983, 49:353-365

178. Dessein AJ, Parker WL, James SL, David JR: IgE antibody and resistance to infection. I. Selective suppression of the IgE antibody response in rats diminishes the resistance and the eosinophil response to Trichinella spiralis infection. J Exp Med 1981, 153:423-436

179. Pritchard DI: Immunity to helminths: is too much IgE parasite--rather than host-protective? Parasite Immunol 1993, 15:5-9

180. Robinson K, Bellaby T, Wakelin D: Immunity to Trichinella spiralis transferred by serum from vaccinated mice not protected by immunization. Parasite Immunol 1995, 17:85-90

181. Ahmad A, Wang CH, Bell RG: A role for IgE in intestinal immunity. Expression of rapid expulsion of Trichinella spiralis in rats transfused with IgE and thoracic duct lymphocytes. J Immunol 1991, 146:3563-3570

182. Else KJ, Grencis RK: Antibody-independent effector mechanisms in resistance to the intestinal nematode parasite Trichuris muris. Infect Immun 1996, 64:2950-2954

183. Jacobson RH, Reed ND, Manning DD: Expulsion of Nippostrongylus brasiliensis from mice lacking antibody production potential. Immunology 1977, 32:867-874

184. McCarthy JS, Moore TA: Enterobiasis. Tropical Infectious Disease: Principles, Pathogens, and Practice. Edited by Guerrant RL, Walker DH, Weller PF. Philadelphia, Elsevier, Churchill Livingstone, 2006, pp 1248-1256

185. University C: General information on nematodes: Enterobius vermicularis, 1998

186. MacPherson DW: Intestinal parasites in returned travelers. Med Clin North Am 1999, 83:1053-1075

187. Saxena AK, Springer A, Tsokas J, Willital GH: Laparoscopic appendectomy in children with Enterobius vermicularis. Surg Laparosc Endosc Percutan Tech 2001, 11:284-286

188. Dickson R, Awasthi S, Demellweek C, William P: Antihelminthic drugs for treatring worms in children: effects on the growth and cognitive performance. Cochrane Database Syst Rev 2003, 2:CD000371

189. Prevention Ca: Pinworm lifecycle

190. Jepps MW, Dobell C: Dientamoeba fragilis n.g, n. sp., new intestinal amoeba from man. Parasitology 1918, 10:352-367

191. Colea A, Silard R, Panaitescu D, Florescu P, Roman N, Capraru T: Studies on Dientamoeba fragilis in Romania. II. incidence of Dientamoeba fragilis in healthy persons. Arch Roum Pathol Exp Microbiol 1980, 39:49-53

192. Katz DE, Taylor DN: Parasitic infections of the gastrointestinal tract. Gastroenterol Clin North Am 2001, 30:797-815, x

193. Dobell C: Researches on the intestinal protozoa of monkeys and man. X. The life history of Dientamoeba fragilis: observations, experiments, and speculations. Parasitology 1940, 32:417-461

194. Burrows RB, Swerdlow MA: Enterobius vermicularis as a probable vector of Dientamoeba fragilis. Am J Trop Med Hyg 1956, 5:258-265

195. Ockert G: [Epidemiology of Dientamoeba fragilis Jepps and Dobell, 1918. 2. Attempt at species transfer with Enterobius eggs]. J Hyg Epidemiol Microbiol Immunol 1972, 16:213-221

196. Ockert G: [Epidemiology of Dientamoeba fragilis Jepps and Dobell, 1918. 3. Further studies on Enterobius transmission through eggs]. J Hyg Epidemiol Microbiol Immunol 1975, 19:17-21

197. Taffs LF: Pinworm infections in laboratory rodents: a review. Lab Anim 1976, 10:1-13

198. Chan KF: Life cycle studies on the nematode Syphacia obvelata. Am J Hyg 1952, 56:14-21

199. Flynn RJ: Nematode. Parasites of laboratory animals. Ames, The Iowa State Univeristy State Press, 1973, pp 238-239

200. Mathies AW: The influence of sex on mouse pinworm infection. Journal of Parasitology 1954, 40:702

201. Eaton GJ: Intestinal helminths in inbred strains of mice. Lab Anim Sci 1972, 22:850-853

202. Panter HC: Studies on host-parasite relationships. Syphacia obvelata in the mouse. Journal of Parasitology 1969, 55:74-78

203. Keeling JE: Experimental trichuriasis. I. Antagonism between Trichuris muris and Aspiculuris tetraptera in the albino mouse. J Parasitol 1961, 47:641-646

204. Sato Y, Ooi HK, Nonaka N, Oku Y, Kamiya M: Antibody production in Syphacia obvelata infected mice. J Parasitol 1995, 81:559-562

205. Beattie GM, Baird SM, Lipsick JS, Lannom RA, Kaplan NO: Induction of T- and B-lymphocyte responses in antigenically stimulated athymic mice. Cancer Res 1981, 41:2322-2327

206. Hoag WG: Oxyuriasis in laboratory mouse colonies. Am J Vet Res 1961, 22:150-153

207. Fratta ID, S. CA: The treatment of oxyurid infested mice with piperazine citrate, stylomycin, caricide, phenothiazine, phthalofyne and gibberellic acid. Proceedings Animal Care Panel 1958, 8:141-146

208. Habermann RT, Fletcher PW, Jr.: The identification and control of helminths in laboratory animals. J Natl Cancer Inst 1958, 20:979-1009

209. Tuffery AA, M IJR: Animals for research: principles of breeding and management. Diseases of laboratory mice and rats. Edited by Lane-Petter W. London and New York, Academic Press, 1963, pp 48-108

210. Hoag WG, Meier H: Biology of the laboratory mouse. Infectious diseases. Edited by Green EL. New York, McGraw Hill, 1966, pp 589-600

211. Harwell JF, Boyd DD: Naturally occurring oxyuriasis in mice. J Am Vet Med Assoc 1968, 153:950-953

212. Jacobson RH, Reed ND: The thymus dependency of resistance to pinworm infection in mice. Journal of Parasitology 1974, 60:976-979

213. Agersborg SS, Garza KM, Tung KS: Intestinal parasitism terminates self tolerance and enhances neonatal induction of autoimmune disease and memory. Eur J Immunol 2001, 31:851-859

214. Huang S, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, Vilcek J, Zinkernagel RM, Aguet M: Immune response in mice that lack the interferon-gamma receptor. Science 1993, 259:1742-1745

215. Magram J, Sfarra J, Connaughton S, Faherty D, Warrier R, Carvajal D, Wu CY, Stewart C, Sarmiento U, Gately MK: IL-12-deficient mice are defective but not devoid of type 1 cytokine responses. Ann N Y Acad Sci 1996, 795:60-70

216. Noben-Trauth N, Kohler G, Burki K, Ledermann B: Efficient targeting of the IL-4 gene in a BALB/c embryonic stem cell line. Transgenic Res 1996, 5:487-491

217. McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, Zurawski G, Murray R, Grencis R, McKenzie AN: Impaired development of Th2 cells in IL-13-deficient mice. Immunity 1998, 9:423-432

218. McKenzie GJ, Fallon PG, Emson CL, Grencis RK, McKenzie AN: Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. J Exp Med 1999, 189:1565-1572

219. Discombe G: Criteria of eosinophilia. Lancet 1946, 1:195

220. Strath M, Warren DJ, Sanderson CJ: Detection of eosinophils using an eosinophil peroxidase assay. Its use as an assay for eosinophil differentiation factors. J Immunol Methods 1985, 83:209-215

221. Huntley JF, Gooden C, Newlands GF, Mackellar A, Lammas DA, Wakelin D, Tuohy M, Woodbury RG, Miller HR: Distribution of intestinal mast cell proteinase in blood and tissues of normal and Trichinella-infected mice. Parasite Immunol 1990, 12:85-95

222. Colley DG, LoVerde PT, Savioli L: Infectious disease. Medical helminthology in the 21st century. Science 2001, 293:1437-1438

223. Reuter JD, Dysko RC: Quality Assurance/Surveilance Monitoring Programs for Rodent Colonies. Laboratory Animal Medicine and Management. Edited by Suckow JDR. Ithaca, New York, International Veterinary Information Service, 2003

224. Luca RR, Alexandre SR, Margues T: Manual Tecnicos em Bioterismo. Sao Paulo, Winner Graph, 1996, pp 259

225. Seder RA: Acquisition of lymphokine-producing phenotype by CD4+ T cells. J Allergy Clin Immunol 1994, 94:1195-1202

226. Dai WJ, Hemphill A, Waldvogel A, Ingold K, Deplazes P, Mossmann H, Gottstein B: Major carbohydrate antigen of Echinococcus multilocularis induces an immunoglobulin G response independent of alphabeta+ CD4+ T cells. Infect Immun 2001, 69:6074-6083

227. Gottstein B: Echinococcus multilocularis infection: immunology and immunodiagnosis. Adv Parasitol 1992, 31:321-380

228. Gottstein B, Hemphill A: Immunopathology of echinococcosis. Chem Immunol 1997, 66:177-208

229. Bresson-Hadni S, Liance M, Meyer JP, Houin R, Bresson JL, Vuitton DA: Cellular immunity in experimental Echinococcus multilocularis infection. II. Sequential and comparative phenotypic study of the periparasitic mononuclear cells in resistant and sensitive mice. Clin Exp Immunol 1990, 82:378-383

230. Devouge M, Ali-Khan Z: Intraperitoneal murine alveolar hydatidosis: relationship between the size of the larval cyst mass, immigrant inflammatory cells, splenomegaly and thymus involution. Tropenmed Parasitol 1983, 34:15-20

231. Emery I, Liance M, Deriaud E, Vuitton DA, Houin R, Leclerc C: Characterization of T-cell immune responses of Echinococcus multilocularis-infected C57BL/6J mice. Parasite Immunol 1996, 18:463-472

232. Mond JJ, Lees A, Snapper CM: T cell-independent antigens type 2. Annu Rev Immunol 1995, 13:655-692

233. Mond JJ, Vos Q, Lees A, Snapper CM: T cell independent antigens. Curr Opin Immunol 1995, 7:349-354

234. Bachmann MF, Zinkernagel RM, Oxenius A: Immune responses in the absence of costimulation: viruses know the trick. J Immunol 1998, 161:5791-5794

235. Amsbaugh DF, Hansen CT, Prescott B, Stashak PW, Barthold DR, Baker PJ: Genetic control of the antibody response to type 3 pneumococcal polysaccharide in mice. I. Evidence that an X-linked gene plays a decisive role in determining responsiveness. J Exp Med 1972, 136:931-949

236. Sieckmann DG, Scher I, Asofsky R, Mosier DE, Paul WE: Activation of mouse lymphocytes by anti-immunoglobulin. II. A thymus-independent response by a mature subset of B lymphocytes. J Exp Med 1978, 148:1628-1643

237. Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J: Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. J Immunol 1999, 163:6448-6454

238. Mohrs M, Holscher C, Brombacher F: Interleukin-4 receptor alpha-deficient BALB/c mice show an unimpaired T helper 2 polarization in response to Leishmania major infection. Infect Immun 2000, 68:1773-1780

239. Cunningham AF, Serre K, Toellner KM, Khan M, Alexander J, Brombacher F, MacLennan IC: Pinpointing IL-4-independent acquisition and IL-4-influenced maintenance of Th2 activity by CD4 T cells. Eur J Immunol 2004, 34:686-694

240. Seki N, Miyazaki M, Suzuki W, Hayashi K, Arima K, Myburgh E, Izuhara K, Brombacher F, Kubo M: IL-4-induced GATA-3 expression is a time-restricted instruction switch for Th2 cell differentiation. J Immunol 2004, 172:6158-6166

241. Finkelman FD, Katona IM, Urban JF, Jr., Holmes J, Ohara J, Tung AS, Sample JV, Paul WE: IL-4 is required to generate and sustain in vivo IgE responses. J Immunol 1988, 141:2335-2341

242. Lawrence RA, Gray CA, Osborne J, Maizels RM: Nippostrongylus brasiliensis: cytokine responses and nematode expulsion in normal and IL-4-deficient mice. Exp Parasitol 1996, 84:65-73

243. Hartmann S, Lucius R: Modulation of host immune responses by nematode cystatins. Int J Parasitol 2003, 33:1291-1302

244. Dainichi T, Maekawa Y, Ishii K, Zhang T, Nashed BF, Sakai T, Takashima M, Himeno K: Nippocystatin, a cysteine protease inhibitor from

Nippostrongylus brasiliensis, inhibits antigen processing and modulates antigen-specific immune response. Infect Immun 2001, 69:7380-7386

245. Newlands GF, Skuce PJ, Knox DP, Smith WD: Cloning and expression of cystatin, a potent cysteine protease inhibitor from the gut of Haemonchus contortus. Parasitology 2001, 122:371-378

246. Rhoads ML, Fetterer RH, Hill DE, Urban JF, Jr.: Trichuris suis: a secretory chymotrypsin/elastase inhibitor with potential as an immunomodulator. Exp Parasitol 2000, 95:36-44

247. Hawley JH, Martzen MR, Peanasky RJ: Proteinase inhibitors in Ascarida. Parasitol Today 1994, 10:308-313

248. Nguyen TT, Qasim MA, Morris S, Lu CC, Hill D, Laskowski M, Jr., Sakanari JA: Expression and characterization of elastase inhibitors from the ascarid nematodes Anisakis simplex and Ascaris suum. Mol Biochem Parasitol 1999, 102:79-89

249. Milstone AM, Harrison LM, Bungiro RD, Kuzmic P, Cappello M: A broad spectrum Kunitz type serine protease inhibitor secreted by the hookworm Ancylostoma ceylanicum. J Biol Chem 2000, 275:29391-29399

250. Hartmann S, Kyewski B, Sonnenburg B, Lucius R: A filarial cysteine protease inhibitor down-regulates T cell proliferation and enhances interleukin-10 production. Eur J Immunol 1997, 27:2253-2260

251. Koninkx JF, Mirck MH, Hendriks HG, Mouwen JM, van Dijk JE: Nippostrongylus brasiliensis: histochemical changes in the composition of mucins in goblet cells during infection in rats. Exp Parasitol 1988, 65:84-90

252. Loke P, MacDonald AS, Allen JE: Antigen-presenting cells recruited by Brugia malayi induce Th2 differentiation of naive CD4(+) T cells. Eur J Immunol 2000, 30:1127-1135

253. Betts J, deSchoolmeester ML, Else KJ: Trichuris muris: CD4+ T cell-mediated protection in reconstituted SCID mice. Parasitology 2000, 121 Pt 6:631-637

254. Artis D: New weapons in the war on worms: identification of putative mechanisms of immune-mediated expulsion of gastrointestinal nematodes. Int J Parasitol 2006, 36:723-733

255. Datta R, deSchoolmeester ML, Hedeler C, Paton NW, Brass AM, Else KJ: Identification of novel genes in intestinal tissue that are regulated after infection with an intestinal nematode parasite. Infect Immun 2005, 73:4025-4033

256. Knight PA, Pemberton AD, Robertson KA, Roy DJ, Wright SH, Miller HR: Expression profiling reveals novel innate and inflammatory responses in the jejunal epithelial compartment during infection with Trichinella spiralis. Infect Immun 2004, 72:6076-6086

257. Komiya T, Tanigawa Y, Hirohashi S: Cloning of the novel gene intelectin, which is expressed in intestinal paneth cells in mice. Biochem Biophys Res Commun 1998, 251:759-762

258. Steppan CM, Brown EJ, Wright CM, Bhat S, Banerjee RR, Dai CY, Enders GH, Silberg DG, Wen X, Wu GD, Lazar MA: A family of tissue-specific resistin-like molecules. Proc Natl Acad Sci U S A 2001, 98:502-506

259. Gruber AD, Gandhi R, Pauli BU: The murine calcium-sensitive chloride channel (mCaCC) is widely expressed in secretory epithelia and in other select tissues. Histochem Cell Biol 1998, 110:43-49

260. Gyomorey K, Garami E, Galley K, Rommens JM, Bear CE: Non-CFTR chloride channels likely contribute to secretion in the murine small intestine. Pflugers Arch 2001, 443 Suppl 1:S103-106

261. Leverkoehne I, Gruber AD: The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. J Histochem Cytochem 2002, 50:829-838

Chapter II

Pinworm influences Ova-induced anaphylaxis

Table of contents

2.1 Summary 106

2.2 Introduction 107

2.2.1 Increasing prevalence of atopic disease 107
2.2.2 Allergy 109
2.2.2.1 Hypersensitivity – Type I 109
2.2.2.1.1 Anaphylaxis 109
2.2.2.1.2 Asthma 111
2.2.2.2 Hypersensitivity – Type II 111
2.2.2.3 Hypersensitivity – Type III 111
2.2.2.4 Hypersensitivity – Type IV 112
2.2.3 Characteristics of allergens 113
2.2.4 Mouse model 113
2.2.4.1 Pathways of systemic anaphylaxis in the mouse 113
2.2.5 Hypothesis 116

2.3 Material and Methods 117

2.3.1 Mice 117
2.3.2 Sensitization 117
2.3.3 Temperature curve 117
2.3.4 Measurement of Ova-specific antibodies 118
2.3.5 Measurement of mouse mast cell proteases-1 118
2.3.6 *Syphacia obvelata* antigen 118
2.3.7 Assessment of cytotoxicity 118
2.3.8 Statistics 118

2.4 Results 119

2.4.1 Pinworm infection has enhances Ova-induced anaphylaxis 119

2.4.2 *S. obvelata* infection does not increase Ova-specific IgE and IgG1 antibody production 119

2.4.3 Ovalbumin primed mice concurrently infected with *S. obvelata* do not induce increased mast cell degranulation 120

2.4.4 *S. obvelata* infection induces non-protective immune response to Ova 123

2.4.5 *S. obvelata* antigen induces a cytotoxic and inhibitory effect on cells 123

2.5 Discussion 126

2.6 Reference 129

2.1 Summary

The prevalence of allergic disease and asthma has increased in the developed world over the last 20 years. A phenomenon almost unheard of in developing countries is now emerging as health facilities improve within rural communities. Developing countries where helminth infections are endemic appear to have reduced allergic disease, which have recently been supported by epidemiological and clinical studies suggesting an inverse association between helminth and atopy. However, studies showing no decrease and in some incidences higher allergy amongst helminth-infected individuals should not be ignored. In our study, we examine the influence of the common GI nematode, *Syphacia obvelata* on a model of anaphylaxis. BALB/c mice sensitized with ovalbumin (Ova) plus alum demonstrated a significant decrease in temperature when challenged on day 28 postinfection. The response was more pronounced in mice infected with *S. obvelata* with a significant decrease in body temperature and increased mortality compared to non-infected mice. Ova-specific IgE and IgG1 were elevated above residue levels induced by PBS plus alum, but showed no difference between infected and non-infected mice. Similarly, no difference in mast cell degranulation was observed between the Ova sensitized groups. Notably however, was the significant reduction of Th2 cytokines IL-4 and IL-5 in mice concomitantly infected with pinworm. Further analysis of *S. obvelata* antigen revealed both an inhibitory and cytotoxic effect which induced a non-antigen specific response. Taken together, these results demonstrate that *S. obvelata* induces a non-protective immune response to Ova-induced anaphylaxis.

2.2 Introduction

2.2.1. Increasing prevalence of atopic disease

The prevalence of allergic disease and asthma has increased in the developed world over the last 20 years, which is not reflected in developing countries [1]. Clear differences have also been noted between urban and rural areas within some countries. Presently, the most consistent hypotheses to explain the increasing prevalence of the dysregulated mucosal Th2 responses that characterize allergic hypersensitivity have been framed in terms of reduced or absent stimulation by Th1-polarized stimuli [2]. Often referred to as the 'hygiene hypothesis' this model suggests that the lack of early childhood bacterial and viral infections brought about by improvement in vaccination and sanitation [3,4] associates with atopy. However, the elimination of chronic Th2-inducing helminth infections, during the same time period, is virtually ignored by this hypothesis. Developing countries where helminth infections are endemic are known to have reduced allergic disease, which have recently been supported by epidemiological and clinical studies suggesting an inverse association between helminth and atopy [5,6]. However, exceptions have also been documented showing helminth infections exacerbate allergic disease. These findings suggest that a common genetic predisposition in some individuals could render them more susceptible to helminth infection and less liable to develop allergies [7].

Studies that have found no decrease and in some incidences higher allergy amongst helminth-infected individuals [5] are also of concern. Although some heterogeneity in the outcome to various helminth parasite species is expected, Yazdanbakhsh and colleagues, argues that intensity and continuity of infection are the important factors influencing the progression to allergy [7]. Low intensity infections can non-specifically potentiate the synthesis of IgE antibody against environmental allergens, and thus enhance allergic reactivity [8-11]. In contrast, heavily infected individuals induce excessive polyclonal IgE which suppresses allergic responses by saturating IgE receptors on mast cells [12] and inhibiting specific IgE synthesis [13,14]. Recent findings

suggest a role for regulatory T cells (Treg, $CD4^{+}CD25^{+}$ T cells) in the suppression of allergy in mice infected with the GI nematode, *H. polygyrus* [15]. In two experimental mouse models of airway allergy, *H. polygyrus* infected BALB/c mice sensitized with ovalbumin and C57BL/6 mice sensitized with house dust mite allergen Derp1, respectively demonstrated depression of airway allergy. Further analysis revealed that the protective effect was retarded in mice treated with anti-CD25 antibody suggesting the involvement of Treg cells [15]. To investigate their hypothesis the authors adoptively transferred $CD4^{+}$ $CD25^{+}$ T cells from worm infected, allergen-naïve animals to uninfected, allergen-sensitized animals. Here, $CD4^{+}$ $CD25^{+}$ T cells transferred suppression of allergy with both antigen-specific and polyclonal IgE antibody levels similar to control mice [15]. Their result supports Treg and not saturation of FcεRI receptors on mast cells to induce suppressive allergic responses. However, it is important to note that studies with GI nematodes *Strongyloides stercoalis* and *Nippostrongylus brasiliensis* as well as others has not eliminated the possibility that saturation of antigen-specific IgE receptors (FcεRI receptors) on mast cells do not play a role in suppression of allergic reactions.

Although IL-10 is known to be involved in many facets of allergy downregulation [16], the role this cytokine plays in the regulation of allergy remains elusive. Presently, it is believed that the increased production of IL-10 by chronic helminth infections downregulate inflammation induced by allergy. IL-10 and transforming growth factor-β (TGF-β) are two anti-inflammatory cytokines that are upregulated in human filariasis and schistosomiasis [17-19].

Here, we examine whether the polarized Th2 response induced by intestinal helminth infection in early life, influences the development of an allergic response to a food allergen.

2.2.2 Allergy

Allergies are reproducible reactions of the immune system against stimuli which are innocuous to most people [20]. They induce an excessive or inappropriate immune response, termed hypersensitivity, which produce tissue damage and may cause severe disease. Hypersensitivity reactions were classified into four types by Gell and Coombs, and correlates clinical symptoms with immunogenic events that occur during allergic reactions [21].

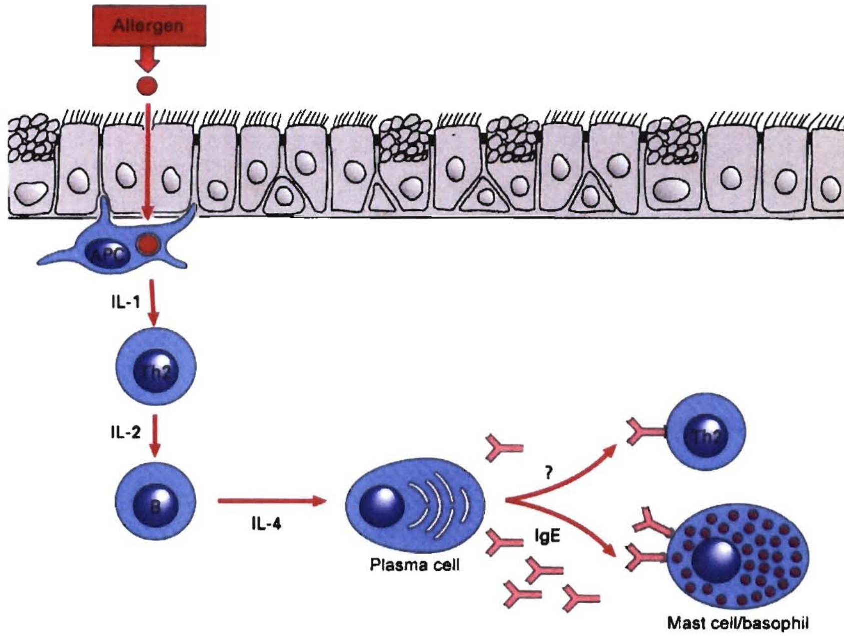
2.2.2.1 Hypersensitivity - Type I

Type I or immediate hypersensitivity (Fig. 2.2) is triggered by antigen cross-linking preformed IgE antibody that is bound to high affinity IgE receptors (FcεRI) on the surface of mast cells and basophils [22]. Conditions associated with Type I hypersensitivity include anaphylaxis and asthma.

2.2.2.1.1 Anaphylaxis

Anaphylaxis, a severe and rapid allergic reaction, is a systemic disorder of immediate hypersensitivity caused by an allergen-specific IgE release of mediators from mast cells and basophils [22]. It consists of two phases. Upon initial exposure of an allergen the sensitization phase is induced (Fig. 2.1A). Genetic and environmental factors promote a Th2-type response with an excess of specific IgE production. These IgE antibodies circulate and rapidly bind FcεRI on mast cells and basophils. A second exposure of the allergen induces the effector phase (Fig. 2.1B). At this point, recognized allergen bound by IgE trigger mast cell degranulation, which leads to the release of histamine and other pharmacological mediators [22].

(A)



(B)

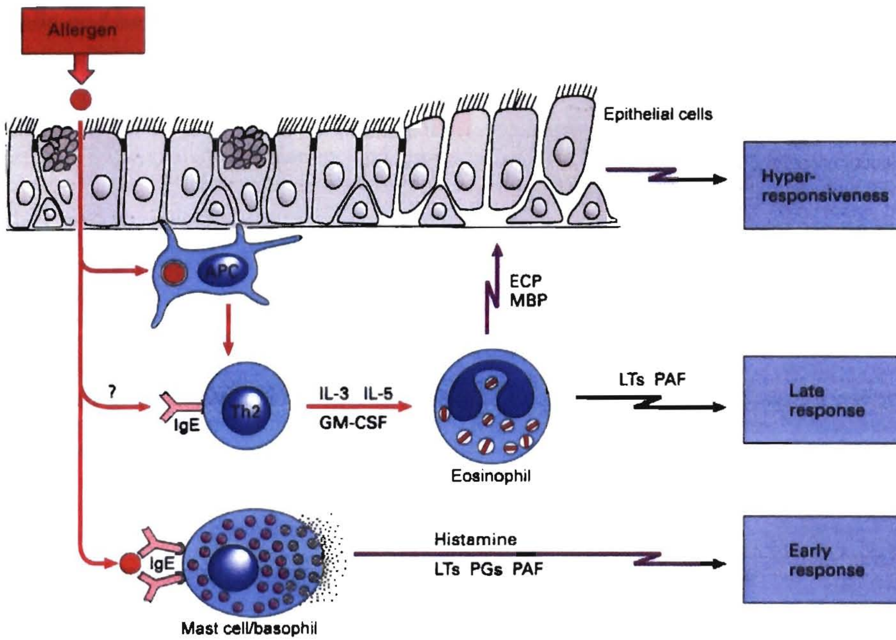


Figure 2.1: Simplified schematic of an anaphylactic reaction. **(A)** The allergic sensitization upon initial exposure of the allergen and **(B)** allergic reaction to a subsequent exposure of the same allergen. This figure has been adapted from Mygind et al. [23]. APC, antigen presenting cell; IL, interleukin; Ig, immunoglobulin; GM-CSF, granulocyte macrophage-colony stimulating factor; ECP, eosinophil cationic protein; MBP, major basic protein; LT, leukotriene; PAF, platelet-activating factor; PG, prostaglandin.

2.2.2.1.2 Asthma

Asthma is a chronic and debilitating disease, causing swollen and inflamed airways that are prone to constrict suddenly and violently [4]. Asthmatic patients are often atopic, with a genetically increased probability of developing IgE hypersensitivities [24]. During an asthmatic attack, smooth muscle cells surrounding the airways contract in a response called acute hyperreactivity, mucus-producing goblet cells secrete excessive amounts of mucus, and the immigration of inflammatory cells into the lungs leads to edema and swelling [20]. These mechanisms narrow the airways, limit air flow and cause breathing problems. Episodes of repeated asthma lead to tissue remodeling with collagen deposition, hypertrophy of smooth muscle and shedding of epithelia [25]. This continual reconstruction makes the lung gradually dysfunctional and may lead to death [20].

2.2.2.2 Hypersensitivity - Type II

Type II or antibody-dependent cytotoxic hypersensitivity occurs when antibodies are produced against self surface or tissue antigens (Fig. 2.2). These antibodies can trigger cytotoxic reactions by stimulating the complement pathway or by facilitating the binding of natural killer (NK) cells [21]. A classic example of this type of hypersensitivity occurs when a person receives a blood transfusion from a donor with a different blood group [26].

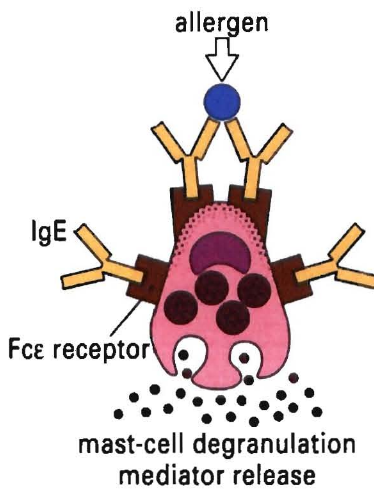
2.2.2.3 Hypersensitivity - Type III

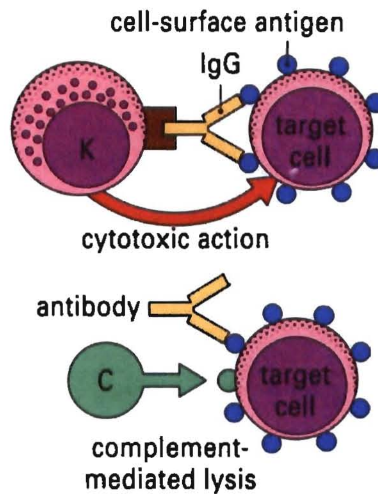
Type III or immune complex disease occurs when normally removed complexes reach excessive amounts and cannot be cleared by macrophages or other cells in the reticuloendothelial system (Fig. 2.2). The accumulation of complexes can lead to either a complement or cell-mediated local reaction. Classical diseases involved with this type of hypersensitivity are systemic lupus erythematosus (SLE) and serum sickness.

2.2.2.4 Hypersensitivity - Type IV

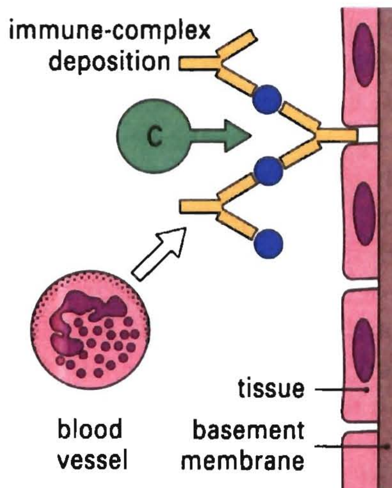
Type IV or cell-mediated reactions involves delayed T cell-mediated immune responses (Fig 2.2). A key characteristic of this hypersensitivity reaction is the time required for the delayed-type hypersensitivity T cells (TDTH, specialized subset of T cells) to migrate to and accumulate near the allergen, which takes a day or more [26].

Type I **Type II**





Type III **Type IV**



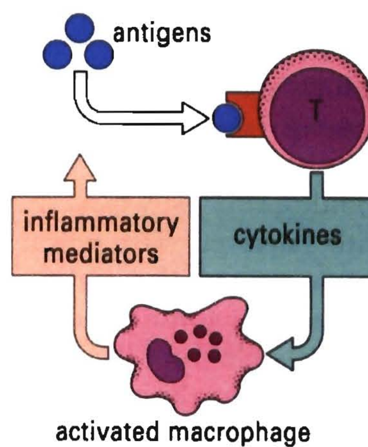


Figure 2.2: Classification of hypersensitivity reactions, type I-IV. The figure has been taken directly from Platts-Mills [21]. IgE, immunoglobulin-E; Fcε receptor, high affinity IgE receptor; K, Killer cells; C, cytotoxic cells; T, Tcell.

2.2.3 Characteristics of allergens

Most allergens are proteins ranging in molecular weights sizes from 10 000 to 40 000 Daltons. They are characteristically soluble in aqueous solutions but have many different biological functions including digestive enzymes, carrier proteins, calycins and pollen recognition proteins [21]. Allergens are classified by their source, route of exposure, and nature of the specific protein.

The food allergen used in this study, ovalbumin (Gal d 2) is a monomeric phosphoglycoprotein with a molecular mass of 43-45kDa and an isoelectic point (pI) of 4.5 [27]. Although initially classified as the major allergen of egg, ovalbumin (Ova) has subsequently been shown to be less frequently recognized by IgE from egg allergic patients [27]. Progress in unraveling the mechanisms of allergic reactions as well as identifying novel hazardous allergens have been made easier with the introduction of animal models. Unfortunately, only few animal models reflecting the allergenicity of proteins in humans are reliable [28].

2.2.4 Mouse model

BALB/c mice are IgE-responding rodents extensively used as a model for allergy studies. Exposed systemically over 3-4 weeks, they produce IgE specific for allergens from food such as peanut, egg, milk and potato [28]. IgE-mediated reactions are the primary basis for most allergies to food, and are a concern because such reactions may lead to death via anaphylaxis [28].

2.2.4.1 Pathways of systemic anaphylaxis in the mouse

Two pathways of systemic anaphylaxis have been demonstrated in murine models. The first, a classical mechanism (IgE-dependent) associated with human allergy is mediated by IgE, FcεRI, mast cells, histamine, and platelet-activating factor (PAF) [29-32]. The second (IgE-independent), mediated by IgG, FcγRIII, macrophages, and PAF

has been associated with allergy in humans, repeatedly exposed to large quantities of antigen.

Murine anaphylaxis induced by either pathway develops and resolve within the same time and have similar symptoms, although subtle differences have been reported in one study [32,33]. IgE-dependent and IgE-independent systemic anaphylaxis are induced by select antigen sensitization and challenge protocols [32]. However, the presence of antigen-specific IgE antibody and mast cells, required for IgE-dependent anaphylaxis, is not the critical factor that determines whether anaphylaxis develops in mice because protocols designed to induce sufficient IgG antibody to allow the induction of IgE-independent anaphylaxis also produce sufficient antigen-specific IgE to mediate IgE-dependent anaphylaxis. Furthermore, the number of mast cells in non-sensitized mice is sufficient to mediate IgE-dependent anaphylaxis [32,34]. Instead, the predominate determinants that influence the induction of IgE-dependent anaphylaxis appear to be the quantity of Ag-specific IgG antibody and the quantity of antigen used to challenge sensitized mice. Thus IgG antibodies, in addition to mediating IgE-independent anaphylaxis, can block IgE-dependent anaphylaxis [35].

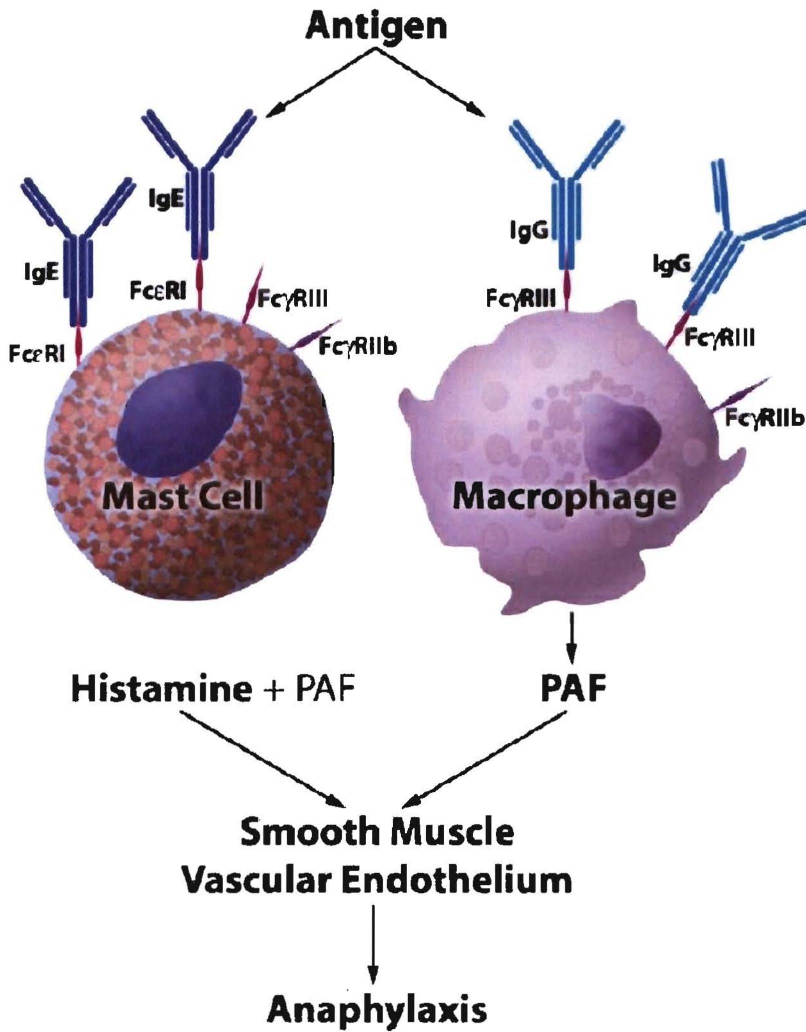


Figure 2.3: Mechanisms of systemic anaphylaxis. This figure was taken directly from Finkelman et al. [35]. Cross-linking of antigen-specific IgE or IgG1 on either mast cells or macrophages result in their degranulation, with the rapid release of mediators such as histamine, PAF and mMCP-1 [32,36]. Ig, immunoglobulin; FcγR, IgG Fc-domains; FcεRI, high affinity IgE receptor ; PAF, platelet activating factor.

2.2.5 Hypothesis

Helminth infections modulate the host immune response thereby influencing the phenotype of a concurrent infection. This has well been documented in the laboratory and field showing reduced allergic disease in helminth infection and endemic areas, respectively. In our study, we investigate whether the asymptomatic pinworm infection skew immune responses to an ovalbumin allergy.

2.3 Material and Methods

2.3.1. Mice

Female BALB/c mice used at 6-8 weeks of age were maintained under specific pathogen free conditions at the animal facility of the Health Science faculty, University of Cape Town (UCT, South Africa).

2.3.2. Sensitization

Pinworm infected and non-infected mice were sensitized on day 14 and 21 with an intraperitoneal (i.p.) injection of 50μg grade V chicken egg OVA (Sigma-Aldrich, USA) and 1.3% aluminum hydroxide (Imject Alum; Pierce, USA) suspended in PBS to a total volume of 200μl. Alum is a Th2 adjuvant which facilitates the slow release of Ova to induce an allergic reaction [37]. Non-sensitized control mice received only PBS plus 1.3% alum. Mice were challenged intravenously on day 28 postinfection with 500μg of Ova per mouse. All mice were bled and tested for *S. obvelata*-specific IgG1 and IgG2b Abs before each experiment. Consequently, mice were bled before the initial sensitization and 50min after the challenge. Pinworm-infected groups are hereafter referred to as Pinworm-infected (Pin) and Pin/Ova-sensitized (Pin/Ova). The non-infected control groups are referred to as Ova (positive control) and PBS (negative control). In each experiment, there were 3-6 mice per group.

2.3.3. Temperature curve

Rectal temperature was measured with a Digital Thermocouple Thermometer (Model BAT-12; Physitery Instruments Inc, NJ, USA) immediately after being challenged, every 5min for 10min, and then every 10 min for the next 50min. At the same time mouse activity was assessed.

2.3.4. Measurement of Ova-specific antibodies

Ova-specific IgE and IgG1 were measure by ELISA as described previously in chapter I.

2.3.5. Measurement of mouse mast cell protease-1

Mouse mast cell protease-1 levels in sera were assayed using a mouse MCP-1 ELISA kit purchased from Moredun Scientific. The ELISA was performed as previously described in chapter I.

2.3.6 *Syphacia obvelata* antigen

Worms isolated from infected mice were isolated, crushed, purified and concentrated as previously described in chapter 1 material and methods. No protease inhibitors were used for antigen preparation.

2.3.7 Assessment of cytotoxicity

Cytotoxicity towards anti-IFN-γ producing hybridoma cells (Clone: R46A2) was estimated by dye exclusion. Briefly, cells were plated in small culture flasks with or without 50μg/ml *Syphacia obvelata* antigen and incubated at 37°C in 5%CO_2. A volume of 45μl hybridoma suspension was then stained with 5μl trypan blue solution (Sigma, USA) and the viabilities expressed as the number of viable cells per 45μl hybridoma supernatant.

2.3.8 Statistics

Data are given as mean ± SD, and the differences were tested using an unpaired Student's *t*-test or ANOVA.

2.4 Results

2.4.1 Pinworm infection enhance Ova-induced anaphylaxis

A pinworm outbreak within one of the SPF animal facilities coincided with some irregularities in mouse experiments, among them ovalbumin (Ova)-induced responses. In order to experimentally investigate if pinworm infection influences experiments, *S. obvelata* infected and non-infected BALB/c mice were sensitized i.p. at day 14 and 21 postinfection with 50μg Ova in alum (Fig. 2.4A). Challenge with 500μg Ova i.v. at day 28 postinfection induced anaphylactic reactions with a marked temperature drop and some mortality in mice (Fig. 2.4B). This phenotype was more pronounced in *S. obvelata* infected mice with consistently greater temperature decline than their non-infected counterparts. Control groups, PBS and Pin showed no change in rectal temperature. The result here shows that *S.obvelata* exacerbates Ova-induced anaphylaxis.

2.4.2 *S. obvelata* infection does not increase Ova-specific IgE and IgG1 antibody production

Murine models elicit 2 pathways of systemic anaphylaxis: one mediated by IgE, FcεRI and mast cells, and the other via IgG, FcγRIII and macrophages [29-32]. To determine whether *S. obvelata* induces an increased production of Ova-specific IgE and IgG1 in mice concomitantly infected and sensitized to Ova allergen, we measured the antibody isotype titers by ELISA. Figure 2.5 shows that sensitization with Ova increased the production of IgE and IgG1 antibody in both Ova and Pin/Ova groups. The response in these groups was markedly elevated above the PBS and Pin control groups. However, *S. obvelata* infection did not increase the production of Ova-specific antibody.

2.4.3 Ovalbumin primed mice concurrently infected with *S. obvelata* does not induce increase mMCP-1

Gastrointestinal nematodes are known to induce increased mMCP-1, in the bloodstream and intestinal lumen of infected mice [38,39]. To determine the hypothesis that increased levels of mMCP-1 correlated with the enhanced hypothermia in *S. obvelata* infected mice sensitized to Ova, we evaluated mast cell degranulation. Quantification of mMCP-1 as a measurement of mast cell degranulation was assayed from mouse sera collected 50min after anaphylactic challenge. Ova sensitized animals showed elevated levels of the mMCP-1 significantly above the PBS control (Fig. 2.6). Mouse sera from BALB/c mice infected only with *S. obvelata* and challenged on day 28 postinfection showed no induction of mMCP-1. This response was confirmed in Ova sensitized groups, as no difference in mMCP-1 levels was observed in the presence (Pin/Ova) or absence (Ova) of *S. obvelata*, suggesting that the helminth does not induce increased degranulation of mast cells.

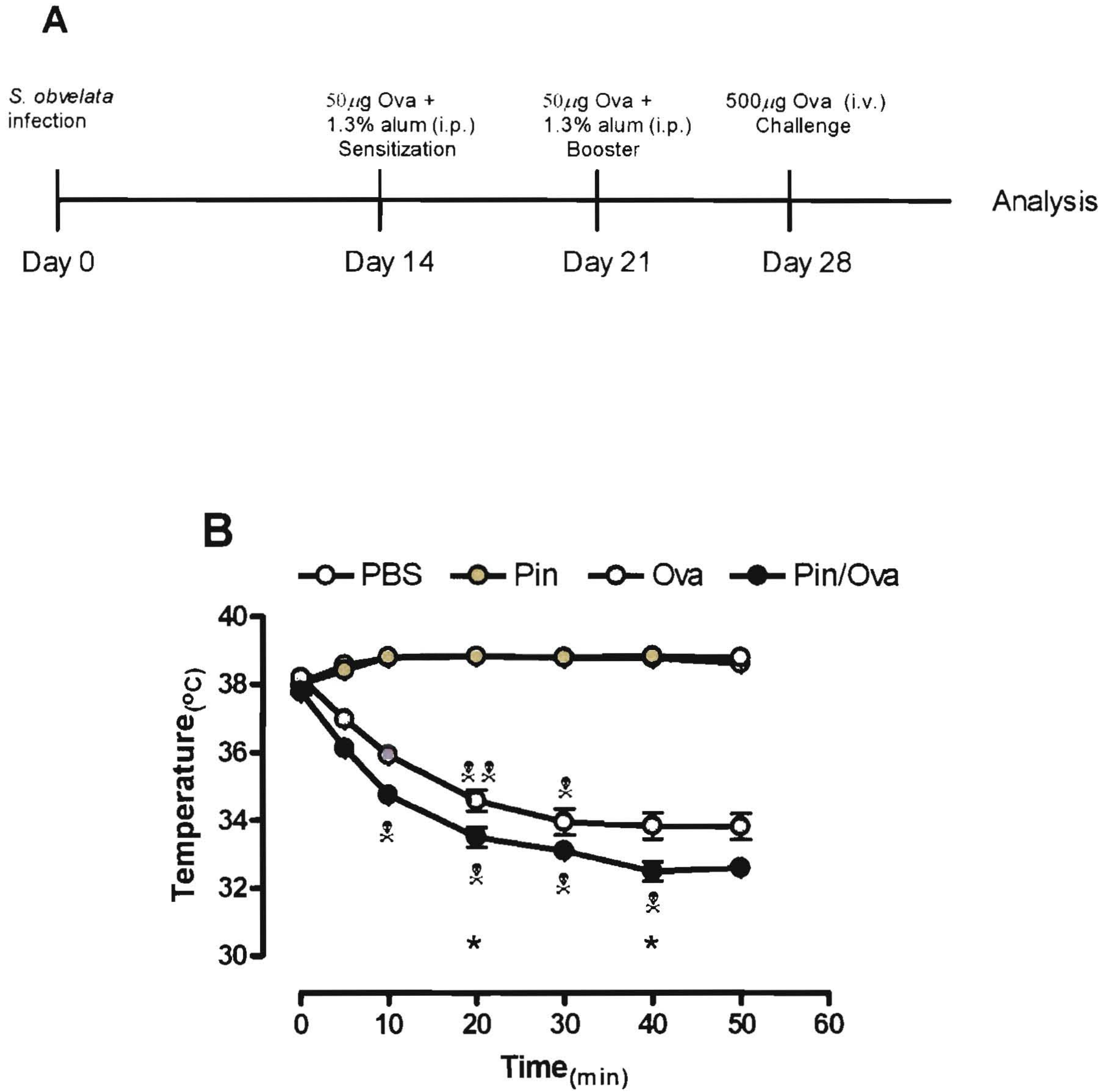


Figure 2.4: *S. obvelata* influences Ova-induced anaphylaxis. **(A)** Schematic representation of the experimental protocol used for intraperitoneal sensitization of BALB/c mice with Ova-antigen and subsequent intravenous challenge. **(B)** *S. obvelata* enhances hypothermia in Ova-induced anaphylaxis. Rectal temperature was measured using a Digital Thermocouple Thermometer immediately after being challenged. The results represent the means ± SD of pooled data from three independent experiments. *, $p < 0.05$, significantly different from Ova group. ☠ ; represents the death of a single mouse.

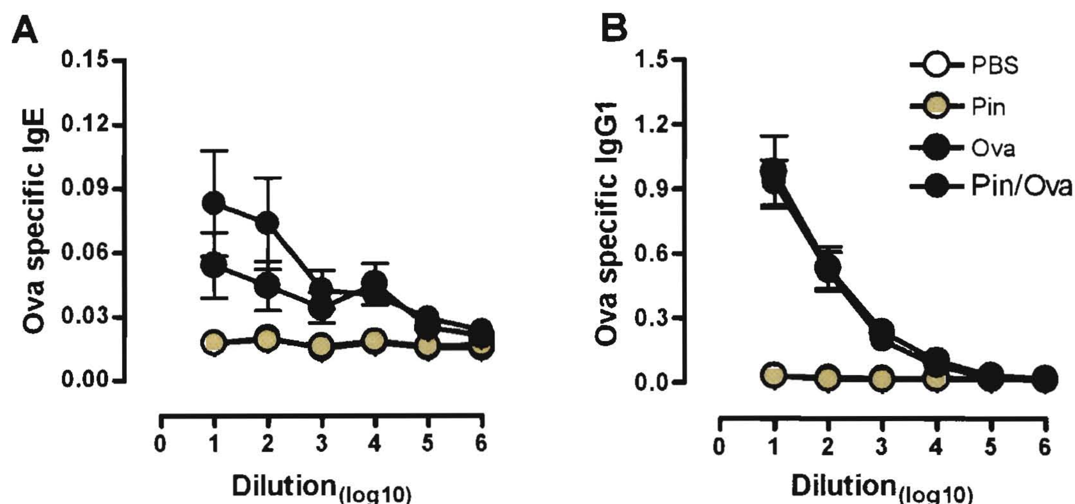


Figure 2.5: Ova-specific IgE **(A)** and IgG1 **(B)** antibody response. Sera from infected and non-infected mice were collected an hour after challenge and analyzed for parasite-specific antibody in an end-point titration ELISA The results are representative of 2 independent experiments. Each point represents the average of four to five mice ± SD.

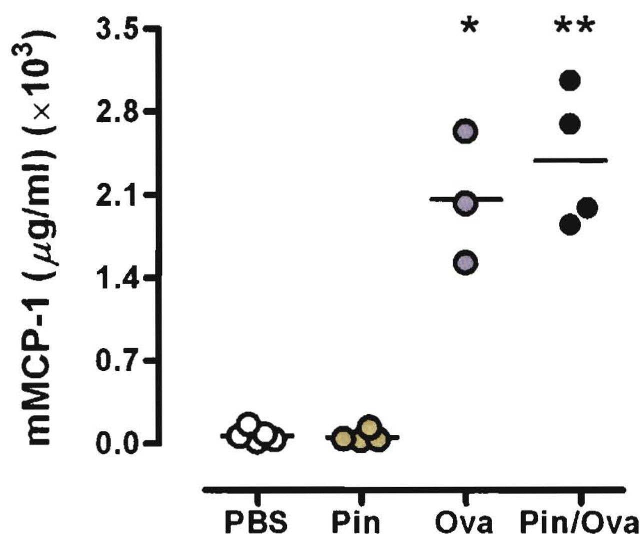


Figure 2.6: Degranulation of mouse mast cell protease-1 (MCP-1). Sera collected prior to the initial sensitization and following the challenged infection were assayed by ELISA for mouse MCP-1. The results are representative of 2 independent experiments. Each symbol represents one mouse.

*, $p < 0.05$; **, $p < 0.01$, significantly different from PBS group.

2.4.4 *S. obvelata* infection induce non-protective immune response to Ova

To examine how the helminth-induced Th2 response might influence allergic sensitization to i.p. administered Ova plus alum, spleen cells from both infected and non-infected mice sensitized with Ova plus alum were harvested and stimulated with Ova *in vitro*. Restimulated spleen cells produced low levels of Ova-specific IFN-γ (Fig. 2.7A) and IL-13 (Fig. 2.7D) cytokines in all groups. Interestingly however, was the marked reduction of Ova-specific IL-4 (Fig. 2.7B) and IL-5 (Fig. 2.7C) in mice infected with pinworm 14 days prior to being sensitized to Ova. This result suggests that *S. obvelata* infection influences Ova-induced cytokine production.

2.4.5 *S. obvelata* antigen induces a cytotoxic and inhibitory effect on cells

We have shown in our anaphylaxis model that a concurrent infection with *S. obvelata* influences experimental results. Noteworthy was the reduced cytokine response of spleen cells from infected mice (Fig. 2.7B and C). To determine the mechanism employed by the GI helminth to reduce cytokine production, anti-IFN-γ producing hybridoma cells were grown in the presence of 50μg/ml parasite antigen. The hybridomas proliferated poorly in the presence of antigen with significantly lower viable cells than the control (Fig. 2.8A). Examination of the cultures revealed an increase in cell debris with a few scattered myeloma cells at 96hrs incubation (Fig 2.8C). In contrast, cultures grown only in media expanded confluently, forming tight cell clusters reminiscent of 'healthy' monoclonal cell growth (Fig. 2.8B). The response here suggests that *S. obvelata* antigen is both cytotoxic and inhibitory of cell growth.

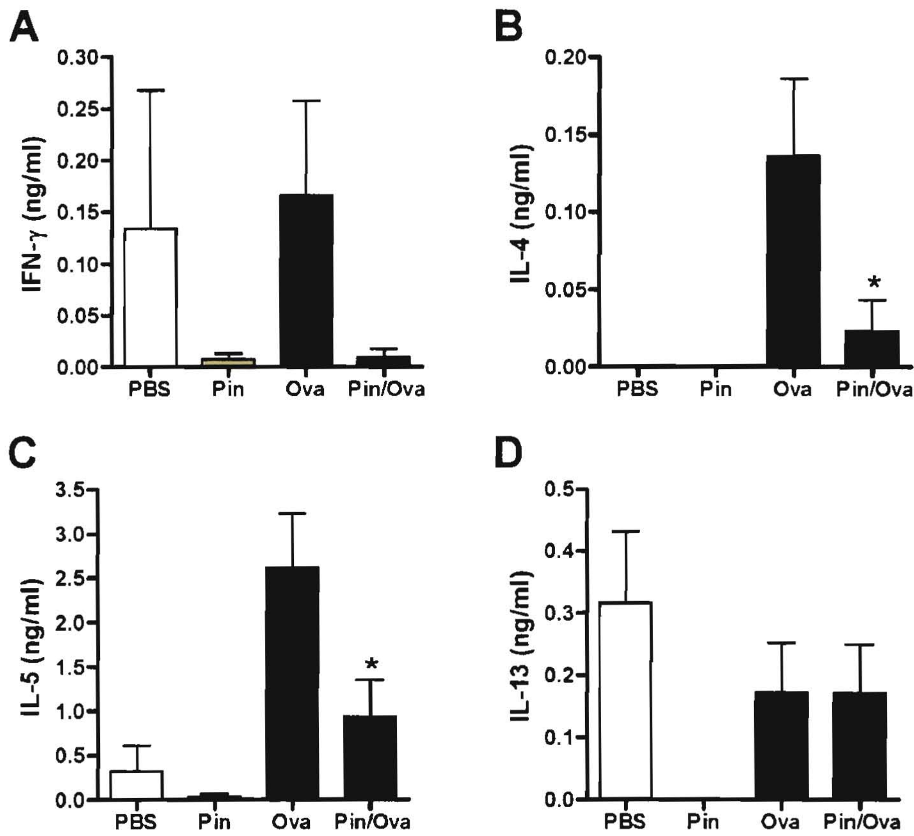


Figure 2.7: *S. obvelata* reduce Ova-specific IL-4 and IL-5 cytokine production. Pooled spleen cells were harvested 21 days after initial sensitization and assayed for Ova-specific IFN-γ **(A)**, IL-4 **(B)**, IL-5 **(C)**, and IL-13 **(D)**. The results represent the means ± SD of pooled data from two independent experiments. n = 4-5 mice.

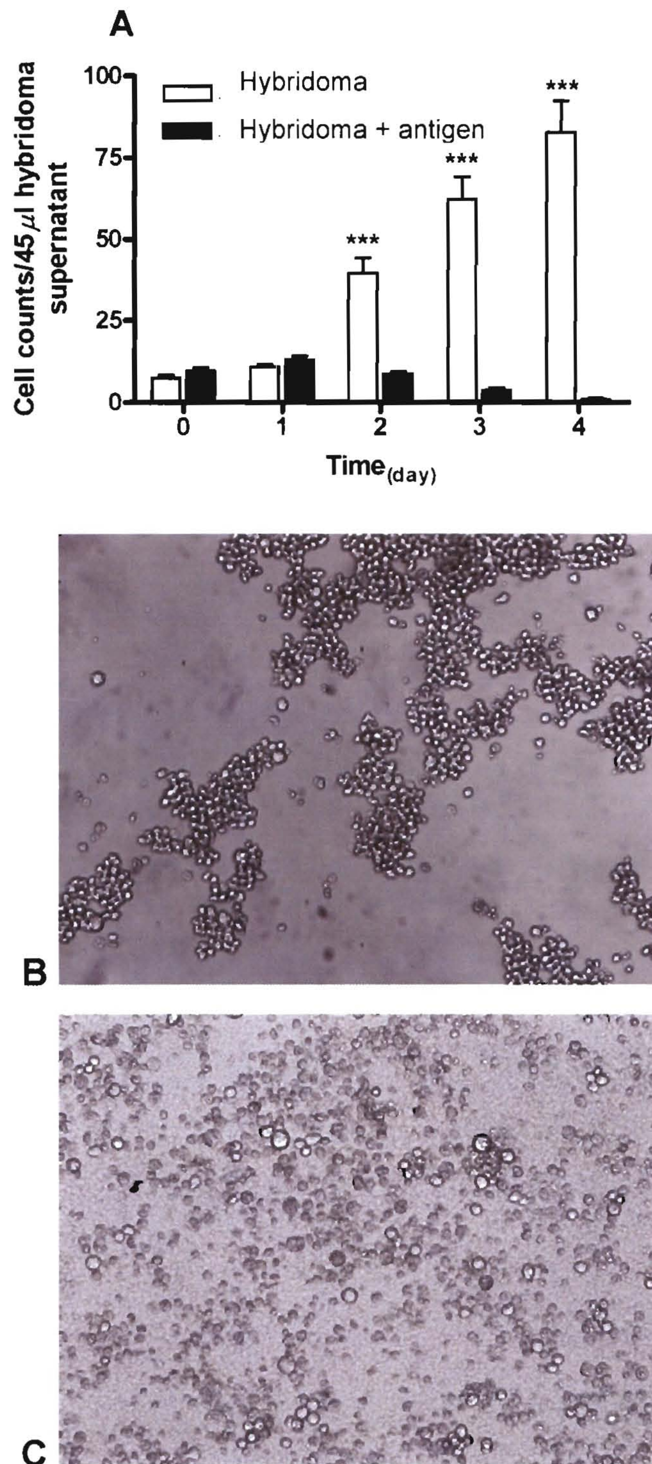


Figure 2.8: *Syphacia obvelata* antigen is non-antigen specific inducing both a cytotoxic and inhibitory effect on cells. **(A)** An estimation of the cytotoxicity toward hybridoma cells by dye exclusion. Open bars represent hybridoma cells without antigen and closed bar present hybridoma cells with antigen. Images on day 4, of anti-IFN-γ producing hybridoma cells were grown in the absence **(B)** or presence **(C)** of 50μg/ml *S. obvelata* antigen. ***, $p < 0.001$, significant different from anti-IFN-γ + antigen.

2.5 Discussion

As infection with pinworm is common in mouse facilities, we investigated whether the GI nematode could influence unrelated experiments. This possibility was confirmed, as BALB/c mice infected with *S. obvelata* responded with a more severe phenotype in experimentally-induced anaphylaxis compared to sham-infected controls. Anaphylactic shock in mice was measured by death and a rapid decline in rectal temperature and activity (activity no shown) which can be induced via IgE, FcεRI and mast cells or IgG1, FcγRII and macrophages [32]. Cross-linking of antigen-specific IgE or IgG1 on either mast cells or macrophages result in their degranulation, with the rapid release of mediators such as histamine, PAF and mMCP-1 [32,36]. In this study, mMCP-1 was measured to indicate the degranulation of the effector cells as the protease was more stable and as it was more cost effective than histamine. Nevertheless, histamine and PAF possibly explain the symptoms observed in response to Ova and should in future be analyzed to access their roles. Previous studies have shown that the vasoactive mediators (histamine and PAF), act on target cells increasing vascular permeability, which causes hemoconcentration by permitting vascular fluid to leak from capillaries and venules, while retaining blood cells [40]. The resulting decrease in vital organ perfusion is the principal cause of symptoms that characterize murine anaphylaxis [34]. *Syphacia obvelata* which induces a Th2-type response (chapter I), enhanced the anaphylactic response to Ova in BALB/c mice, eliciting increased hypothermia and mortality. However, Ova-specific IgE and IgG1 as well as mMCP-1 levels showed no significant difference in Ova sensitized mice in the absence or presence of a worm infection. As a result, the hypothesis that an enhanced Th2 response was stimulated in the presence of both worm and allergy, subsequently resulting in an increase of mast/macrophage degranulation seems less plausible. IL-4 and IL-13 exacerbate anaphylaxis by increasing the sensitivity of vasoactive mediators [34]. However, *S. obvelata* infected mice concomitantly sensitized to Ova plus alum elicited lower levels of Ova-specific IL-4 (Fig. 2.6B) and IL-5 (Fig. 2.6 C), while IL-13 was undetectable (Fig. 2.6D) during *in vitro* restimulation. This observation reiterates that a 'super' Th2 response was not the cause of the enhanced anaphylaxis observed in our model.

The ability of helminths and their antigen to modulate allergic responses has well been documented. The interaction between helminth and allergy depend on a few variables: phase (acute or chronic) of helminth infection, parasite load and species of helminth [41]. Acute helminth infections may increase manifestation of allergy as a result of the Th2 immune response elicited by the worms, suggesting that the helminth predispose individuals to allergic reactions. However, an enhance Th2 response (super Th2) was not induced in mice primed with *S. obvelata* prior to being sensitized to Ova plus alum. This was shown by a reduced Th2 response (reduced Ova-specific IL-4 and IL-5). A heavy parasite load can also be excluded as the cause of immunomodulation because BALB/c mice were shown to harbor low worm burdens throughout the course of an infection.

Donnelly et al. have previously shown that helminth proteases, which exhibit significant homology with known allergens, can manifest allergic reactions in the skin and the respiratory tract [42]. The cysteine protease of house dust mite, Der p1, the aspartic protease of cockroach, Bla g2, the serine protease of *Aspergillus fumigatus* and the bacterial subtilisins are all major allergenic molecules responsible for asthma and atopic disease [42]. These proteases have been documented to induce Th2-driven inflammatory responses in airways by disrupting the epithelial cell junctions so that these, and other molecules, gain access to, and alter the function of, underlying cells of both the innate and adaptive immune system. Helminths produce proteases to gain entry into their hosts, and to feed on and migrate through tissue. Therefore, it should come as no surprise that *S. obvelata* proteases may induce the altered responses experienced in pinworm infected mice concomitantly sensitized to ovalbumin. These molecules may also be responsible for the cytotoxic and inhibitory effects we showed with ant-IFN-γ producing hybridoma cells. The myeloma cells grew poorly in the presence of *S. obvelata* antigen and showed profound cell death from day 3 onward. Like IL-4 which enhance the severity of anaphylaxis by increasing the effects of vasoactive mediators [34], it is possible that *S. obvelata* worms induce the same response, which would induce the hypothermia observed in our study. *Syphacia obvelata* proteases although not identified yet, may have acted directly or indirectly to increase vascular permeability and induce anaphylactic shock. This hypothesis would require further investigation.

Immunosuppressive effects from helminths and inhibition of allergic reactions was previously shown during immunization with *Ascaris suum* extract [43], and *Heligmosomoides polygyrus* [2] infection studies with IL-10 the responsible suppressive factor. The role of IL-10 in downregulating allergy remains elusive but has been associated with reducing inflammation induced by allergy [17-19]. *Syphacia obvelata* induces an early production of IL-10 day 7 postinfection (Fig. 1.11, chapter I) but whether this cytokine is responsible for the reduced Ova-specific IL-4 and IL-5 production would require further investigation.

Proteases inhibitors discussed in chapter I may also account for the reduced cytokine production observed in our allergy model.

Although this could explain the reduced Ova-specific cytokine production, it does not account for the increased cell death of myeloma cells shown in Figure 2.8 (Chapter II). We suspect that the amount of 50μg/ml pinworm antigen might have been toxic and that a titration of the antigen would rule out this possibility. If 50μg/ml pinworm antigen is toxic on cells, it would explain why the mesenteric lymph node and spleen cells stimulated with antigen ex vivo produced such low cytokine levels.

Immune evasion mechanisms used by nematodes to affect host immunity are not uncommon [44] and have been shown in studies with *T. spiralis*, *H. polygyrus* [45-50], *N. brasiliensis* [51], and *A. suum* [52-54]. This ability of nematodes to skew disease outcomes have been highlighted by the hygiene hypothesis with studies showing lower prevalence of allergies in helminth-infected populations [55-57]. However, not all experimental models support a protective response [5], including the observed increased anaphylactic shock in pinworm-infected mice. It is well possible that other factors, like intensity and continuity of infection [7], or the above discussed proteases and protease inhibitors may influence progression to allergy.

2.6 References

1. Kim DS, Drake-Lee AB: Infection, allergy and the hygiene hypothesis: historical perspective. J Laryngol Otol 2003, 117:946-950

2. Bashir ME, Andersen P, Fuss IJ, Shi HN, Nagler-Anderson C: An enteric helminth infection protects against an allergic response to dietary antigen. J Immunol 2002, 169:3284-3292

3. Strachan DP: Hay fever, hygiene, and household size. Bmj 1989, 299:1259-1260

4. Cookson WO, Moffatt MF: Asthma: an epidemic in the absence of infection? Science 1997, 275:41-42

5. Mao XQ, Sun DJ, Miyoshi A, Feng Z, Handzel ZT, Hopkin JM, Shirakawa T: The link between helminthic infection and atopy. Parasitol Today 2000, 16:186-188

6. Weiss ST: Parasites and asthma/allergy: what is the relationship? J Allergy Clin Immunol 2000, 105:205-210

7. Yazdanbakhsh M, van den Biggelaar A, Maizels RM: Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. Trends Immunol 2001, 22:372-377

8. Turner KJ, Feddema L, Quinn EH: Non-specific potentiation of IgE by parasitic infections in man. Int Arch Allergy Appl Immunol 1979, 58:232-236

9. Joubert JR, van Schalkwyk DJ, Turner KJ: Ascaris lumbricoides and the human immunogenic response: enhanced IgE-mediated reactivity to common inhaled allergens. S Afr Med J 1980, 57:409-412

10. Lynch NR, Di Prisco-Fuenmayor MC: High allergic reactivity in a tropical environment. Clin Allergy 1984, 14:233-240

11. Buijs J, Lokhorst WH, Robinson J, Nijkamp FP: Toxocara canis-induced murine pulmonary inflammation: analysis of cells and proteins in lung tissue and bronchoalveolar lavage fluid. Parasite Immunol 1994, 16:1-9

12. Godfrey RC, Gradidge CF: Allergic sensitisation of human lung fragments prevented by saturation of IgE binding sites. Nature 1976, 259:484-486

13. Hagel I, Lynch NR, Di Prisco MC, Rojas E, Perez M, Alvarez N: Ascaris reinfection of slum children: relation with the IgE response. Clin Exp Immunol 1993, 94:80-83

14. Lynch NR, Hagel I, Di Prisco MC, Lopez RI, Garcia NM, Perez M: Serum IgE levels, helminth infection and socioeconomic change. Parasitol Today 1992, 8:166-167

15. Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE: Helminth parasites--masters of regulation. Immunol Rev 2004, 201:89-116

16. Akbari O, DeKruyff RH, Umetsu DT: Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat Immunol 2001, 2:725-731

17. Mahanty S, Nutman TB: Immunoregulation in human lymphatic filariasis: the role of interleukin 10. Parasite Immunol 1995, 17:385-392

18. Doetze A, Satoguina J, Burchard G, Rau T, Loliger C, Fleischer B, Hoerauf A: Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift. Int Immunol 2000, 12:623-630

19. King CL, Medhat A, Malhotra I, Nafeh M, Helmy A, Khaudary J, Ibrahim S, El-Sherbiny M, Zaky S, Stupi RJ, Brustoski K, Shehata M, Shata MT: Cytokine control of parasite-specific anergy in human urinary schistosomiasis. IL-10 modulates lymphocyte reactivity. J Immunol 1996, 156:4715-4721

20. Duschl A: IL-13 in allergy and asthma. Interleukin-13. Edited by Brombacher F. Georgetown, Landes Biosciences, 2003, pp 79-92

21. Platts-Mills T: Hypersensitivity - Type I. Immunology. Edited by Roitt I, Brostoff J, Male D. Edinburgh, Mosby, 2001, pp 324-343

22. Janeway CAJ, Travers P: Allergy and Hypersensitivity. ImmunoBiology: The Immune System in Health and Disease. Edited by Austin P, Lawrence E. New York, Garland Publishing, 1997, pp 11.11-11.25

23. Mygind N, Dahl R, Pedersen S, Thestrup-Pedersen K: Essential Allergy, Blackwell Science, 1996

24. Sandford AJ, Pare PD: The genetics of asthma. The important questions. Am J Respir Crit Care Med 2000, 161:S202-206

25. Bochner BS, Undem BJ, Lichtenstein LM: Immunological aspects of allergic asthma. Annu Rev Immunol 1994, 12:295-335

26. Prescott L, Harley JP, Klein DA: Medical Microbiology. USA, Wm. C. Brown Publishers, 1996

27. Demeulemester C, Giovannacci I: Detecting dairy and egg residues in food. Detecting allergens in food. Edited by Koppelman SJ, Hefle SL. Cambridge, Woodhead Publishing Limited, 2006, pp 219-243

28. Woolhiser MR, Metcalf DD: Safety Issues in Food Biotechnology. Food Alergy: Adverse Reactions to Foods and Food Additives. Edited by Metcalf

DD, Sampson HA, Simon RA. Massachusetts, Blackwell Publishing Company, 2003, pp 71-80

29. Miyajima I, Dombrowicz D, Martin TR, Ravetch JV, Kinet JP, Galli SJ: Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc gammaRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. J Clin Invest 1997, 99:901-914

30. Oettgen HC, Martin TR, Wynshaw-Boris A, Deng C, Drazen JM, Leder P: Active anaphylaxis in IgE-deficient mice. Nature 1994, 370:367-370

31. Bruschi F, Pozio E, Watanabe N, Gomez-Morales MA, Ito M, Huang Y, Binaghi R: Anaphylactic response to parasite antigens: IgE and IgG1 independently induce death in Trichinella-infected mice. Int Arch Allergy Immunol 1999, 119:291-296

32. Strait RT, Morris SC, Yang M, Qu XW, Finkelman FD: Pathways of anaphylaxis in the mouse. J Allergy Clin Immunol 2002, 109:658-668

33. Takeishi T, Martin TR, Katona IM, Finkelman FD, Galli SJ: Differences in the expression of the cardiopulmonary alterations associated with anti-immunoglobulin E-induced or active anaphylaxis in mast cell-deficient and normal mice. Mast cells are not required for the cardiopulmonary changes associated with certain fatal anaphylactic responses. J Clin Invest 1991, 88:598-608

34. Strait RT, Morris SC, Smiley K, Urban JF, Jr., Finkelman FD: IL-4 exacerbates anaphylaxis. J Immunol 2003, 170:3835-3842

35. Finkelman FD, Rothenberg ME, Brandt EB, Morris SC, Strait RT: Molecular mechanisms of anaphylaxis: lessons from studies with murine models. J Allergy Clin Immunol 2005, 115:449-457; quiz 458

36. Dombrowicz D, Flamand V, Brigman KK, Koller BH, Kinet JP: Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene. Cell 1993, 75:969-976

37. Flarend RE, Hem SL, White JL, Elmore D, Suckow MA, Rudy AC, Dandashli EA: In vivo absorption of aluminium-containing vaccine adjuvants using 26Al. Vaccine 1997, 15:1314-1318

38. Huntley JF, Gooden C, Newlands GF, Mackellar A, Lammas DA, Wakelin D, Tuohy M, Woodbury RG, Miller HR: Distribution of intestinal mast cell proteinase in blood and tissues of normal and Trichinella-infected mice. Parasite Immunol 1990, 12:85-95

39. Pemberton AD, Wright SH, Knight PA, Miller HR: Anaphylactic release of mucosal mast cell granule proteases: role of serpins in the differential clearance of mouse mast cell proteases-1 and -2. J Immunol 2006, 176:899-904

40. Jancar S, Sirois MG, Carrier J, Braquet P, Sirois P: PAF induces rat plasma extravasation and releases eicosanoids during anaphylaxis. Inflammation 1991, 15:347-354

41. Carvalho EM, Bastos LS, Araujo MI: Worms and allergy. Parasite Immunol 2006, 28:525-534

42. Donnelly S, Dalton JP, Loukas A: Proteases in helminth- and allergen-induced inflammatory responses. Chem Immunol Allergy 2006, 90:45-64

43. Ferreira AP, Faquim ES, Abrahamsohn IA, Macedo MS: Immunization with Ascaris suum extract impairs T cell functions in mice. Cell Immunol 1995, 162:202-210

44. Barriga OO, Ingalls WL: Potentiation of an IgE-like response to Bordetella bronchiseptica in pigs following Ascaris suum infection. Vet Parasitol 1984, 16:343-345

45. Faubert G, Tanner CE: Trichinella spiralis: inhibition of sheep hemagglutinins in mice. Exp Parasitol 1971, 30:120-123

46. Jones JF, Crandall CA, Crandall RB: T-dependent suppression of the primary antibody response to sheep erythrocytes in mice infected with Trichinella spiralis. Cell Immunol 1976, 27:102-110

47. Barriga OO: Depression of cell-mediated immunity following inoculation of Trichinella spiralis extract in the mouse. Immunology 1978, 34:167-173

48. Shimp RG, Crandall RB, Crandall CA: Heligmosomoides polygyrus (=Nematospiroides dubius): suppression of antibody response to orally administered sheep erythrocytes in infected mice. Exp Parasitol 1975, 38:257-269

49. Losson B, Lloyd S, Soulsby EJL: Nematospiroides dubius: Modulation of the host immunological responsive. Parasitology Research 1985, 71:383-393

50. Kwa BH, M. JW: Immunology, Immunopathology and Immunoprophylaxis of Parasite Infections. Immune Responses in Parasitic Infections. Edited by Soulsby EJL. Florida, USA, CRC Press, 1987, pp 223-249

51. Ishizaka K: Regulation of IgE biosynthesis. Hosp Pract (Off Ed) 1989, 24:51-60

52. Komatsu T, Nishimura T, Sano R, Shinka S: Ascaris suum: suppression of reaginic and hemagglutinating antibody responses in the mouse by crude extract and maintenance fluid. Exp Parasitol 1979, 47:158-168

53. de Macedo MS, Mota I: Antigenic competition in IgE antibody production. I. Establishment of parameters involved in primary and secondary responses. Immunology 1980, 40:701-708

54. Soares MF, Macedo MS, Mota I: Suppressive effect of an Ascaris suum extract on IgE and IgG antibody responses in mice. Braz J Med Biol Res 1987, 20:203-211

55. Hagel I, Lynch NR, DiPrisco MC, Lopez RI, Garcia NM: Allergic reactivity of children of different socioeconomic levels in tropical populations. Int Arch Allergy Immunol 1993, 101:209-214

56. van den Biggelaar AH, van Ree R, Rodrigues LC, Lell B, Deelder AM, Kremsner PG, Yazdanbakhsh M: Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10. Lancet 2000, 356:1723-1727

57. Araujo MI, Lopes AA, Medeiros M, Cruz AA, Sousa-Atta L, Sole D, Carvalho EM: Inverse association between skin response to aeroallergens and Schistosoma mansoni infection. Int Arch Allergy Immunol 2000, 123:145-148

Chapter III

Diagnostic analysis

Table of contents

3.1 Summary 139

3.2 Introduction 140

3.2.1 Diagnostic detection and identification of potential pathogens 140
3.2.1.1 Quantitative analysis 142
3.2.1.2 Antibody-Antigen interaction 144
3.2.1.2.1 Stages involved during antibody binding 144
3.2.1.3 Cross-reactivity 146
3.2.1.3.1 Cross-reactivity of helminths 146
3.2.1.4 Polyclonal antibodies 146
3.2.1.4.1 The advantage and application of polyclonal antibodies 147
3.2.1.5 Generation of monoclonal antibodies (hybridomas) 147
3.2.1.5.1 Application of monoclonal antibodies 149
3.2.2 Hypothesis 150

3.3 Material and Methods 151

3.3.1 Animals 151
3.3.2 Methods of isolating S. obvelata worms and eggs 151
3.3.2.1 Cellophane (Scotch tape) method 151
3.3.2.2 Lewis and D'Silva's method 151
3.3.2.3 Stahl's method 152
3.3.3 Producing an arbitrary standard 152
3.3.4 ELISA 152
3.3.5 Mycoplasma detection ELISA 152
3.3.6 Rabbit polyclonal antibody 153

3.3.6.1 Bleeding from the auricular artery 153
3.3.6.2 Protocol for producing polyclonal antibody 154
3.3.6.3 Quantifying the rabbit polyclonal antibody titer 154
3.3.7 Rat monoclonal antibody 155
3.3.7.1 Feeder layer 155
3.3.7.2 Fusion 155
3.3.7.3 Preparing cells 156
3.3.7.4 Monitor and feed cells 156
3.3.7.5 Screening primary hybridoma supernatants 157
3.3.7.6 Establishing of hybridoma lines 157
3.3.7.7 Cloning by limiting dilution 157
3.3.8 Growing hybridomas for purification 158
3.3.8.1 Purifying antibodies 158
3.3.8.1.1 Preparing the protein G-column 158
3.3.8.1.2 Purifying and selecting IgG isotype antibody on a protein-G Sepharose column 159
3.3.9 Denaturing (SDS) discontinuous gel electrophoresis 159
3.3.9.1 Separating gel 160
3.3.9.2 Stacking gel 160
3.3.9.3 Sample preparation 160
3.3.9.4 Sample loading and gel electrophoresis 161
3.3.10 Immunoblotting and immunodetection (Western blot) 161
3.3.10.1 Assembling of the immunoblot sandwich 161
3.3.10.2 Visualization of *S. obvelata* protein 162

3.4 Results 163

3.4.1 Establishing an indirect pinworm specific ELISA 163

3.4.2 Preparing hyper-immune serum 163

3.4.3 Analyzing the cross-reactivity of anti-*S. obvelata* serum against antigen of other research parasites 166

3.4.4 The efficacy of anti-*S. obvelata* polyclonal antibody in an ELISA 168

3.4.5 The efficacy of anti-*S. obvelata* monoclonal antibody in an ELISA 170

3.4.6 Antigenicity of *S. obvelata* somatic antigen 170

3.5 Discussion 173

3.6 Reference 178

3.1 Summary

With the advance of technology, new specialized mouse strains are rapidly developed and easily transported globally. As a result, overcrowding of research animal facilities is threatened by adventitious infections with the potential of influencing experimental results. It is therefore of utmost importance that surveillance of such threats accurately and rapidly be diagnosed and controlled. Using the Enzyme-linked Immunosorbent assay (ELISA), we established an optimal pinworm-specific antibody detection system, to simply and easily detect the GI nematode in a mouse colony. In this study, we demonstrated that 5μg/ml *Syphacia obvelata* antigen as coating concentration elicited a 1000-fold better binding specificity than either lower or higher concentrations, in an indirect ELISA. We established an arbitrary standard for quantification and showed that the hyper-immune serum is non-specific to most parasite antigens with the exception of *Anisakis pegreffii* and *Nippostrongylus brasiliensis*. We developed rabbit polyclonal anti-*S. obvelata* antibody showing their increased sensitivity in detecting pinworm both in an indirect and direct ELISA. Furthermore, we generated monoclonal antibodies to *S. obvelata* somatic antigen which proved limiting for serodiagnosis. Nonetheless, 4 antigenic proteins were identified for future analysis as potential candidates for detecting *S. obvelata* infection. In conclusion, we have shown that the optimized ELISAs established in this study are sensitive and can be used as diagnostic tools for detecting the GI nematode pinworm.

3.2 Introduction

In the previous chapters we discussed the pervasive problem of pinworm, addressing the immune response induced by the GI nematode and the consequence of such an infection. It is important to emphasize the risks of introducing adventitious infections as the global exchange of animals and animal products increase among laboratories [1]. Equally concerning is the increasing availability of genetically engineered mice, whose responses to infection and disease can be unpredictable, adding to the already overwhelming problem. These specialized mouse strains continue to be developed with the advance of biotechnology, significantly adding to the size of institutional rodent colonies and the complexity of rodent health care [1]. In a study investigating the burden of adventitious pathogens in SPF mouse colonies, the authors reported the presence of infectious agents in 10-35% of the research institutions investigated. Prevalence was as expected, higher among non-SPF mice with pinworm reported in 70% of institutions housing rodents under these conditions. Hence, the prevention and elimination of threatening infections among valuable, densely housed rodents, such as transgenic mice, require regular testing, appropriate housing, rapid diagnosis, and control of possible outbreaks.

3.2.1. Diagnostic detection and identification of potential pathogens

Today various methodologies are used to identify potential pathogens in laboratory animals. The most direct evidence is the culturing of an organism from a tissue or biological specimen. What determines the technique used, depends on a number of factors including the type of organism, the fastidiousness of the organism, the immunological status of the host, the tissue or site of localization of the organism in the host, and the particular tests that have been developed to detect and identify the organism [1]. In the case of pinworm, 3 well known techniques are routinely used. The cellophane tape [2] and Lewis and D'Silva methods [3], the simpler of the 3, are easy and inexpensive.

The disadvantages of these methods are that they require trained staff to identify and isolate the eggs and worms of the nematode. For positive identification, these methods require 3-5 consecutively collected samples taken early in the morning. Furthermore, resistant mouse strains (BALB/c, C57BL/6, 129/Sv) which rapidly expel the worm can easily be misdiagnosed. The third technique, Stahl's method [4], provides direct evidence of pinworm infection by visualizing the worm in the cecum of the host. This method has proven ideal for isolating infective eggs and generating parasite antigen (Ag). As a diagnostic tool however, Stahl's method is least favorable, since it requires mice to be killed for analysis. Although possible, misdiagnoses of pinworm in resistant mice are less likely using this method.

Directly identifying pathogens in their niche would be the standard diagnosis with which to control any potential problem. Nevertheless, this is not always possible and alternative techniques equally sensitive have to be used. Serology, as an analytical tool uses the *in vivo* antibody (Ab) response to measure and detect, *in vitro*, the response to Ags. This method is quite attractive for diagnosing whether mice are or have previously been infected with pinworm. Diseases of the digestive tract as in the case of pinworm infections are not always observed clinically. This can partly be explained as the more serious effects of these pathogens are evident only in the very young i.e. neonates and sucklings [5]. Accordingly, weanlings or young adults (4-6months old) may show no clinical signs when delivered to the researcher [5]. They will however be protected against infections by antibodies passed through their mother's colostrums [6,7]. At weaning, this protection is lost [8] and mice become susceptible to pathogens in their environment. SPF animals ideally should have no protection against infectious agents provided they are housed correctly. The initial immunoglobulin response mounted by infected weanlings is an IgM response, which lasts for days or weeks [9]. This response eventually becomes primarily IgG after the first week or 2 and continues for months or years particularly in the presence of continued antigenic stimulation by infectious agents in its environment [10].

3.2.1.1 Quantitative analysis

The Enzyme-linked Immunosorbent assay (ELISA) is one of the most widely used serological tests for Ab and Ag detection [11]. Of the two basic forms; the direct (sandwich) ELISA is used for the detection of Ag, whilst the indirect ELISA has proven useful in detecting Ab. As a diagnostic tool, the ELISA is simple, flexible, reproducible, sensitive (ng/ml range), uses stable reagents (alkaline phosphatase conjugates typically lose only 5% to 10% of their activity per year) [12], does not require radiation procedures and is relatively inexpensive [13].

The ELISA is a powerful assay which combines the properties of antigen-antibody interactions with simple phase separations to detect biological molecules [12]. The multivalency of antibodies allows the formation of stable Ag-Ab complexes which can easily be measured. Based on the design of the assay, capture reagents initiates the binding of Ag-Ab complexes and enzyme conjugates onto a solid phase. Any unbound reagents are easily washed away allowing the bound conjugate to be visualized by adding substrate which produces a detectable product, when hydrolyzed by the conjugated enzyme. Important is the stability of the enzyme, which continually acts on substrate resulting in the amplification of the reaction [1]. The colored product produced is proportional to the Ab bound within the limits of the optimized assay. Unfortunately, detection of an Ab response generally requires between 1-2 weeks to produce detectable Ab levels [1]. Despite this drawback, pinworm-specific ELISAs will allow researchers to test whether their mice had been exposed to the nematode prior to (under SPF conditions, minimal risk), and after an experimental study.

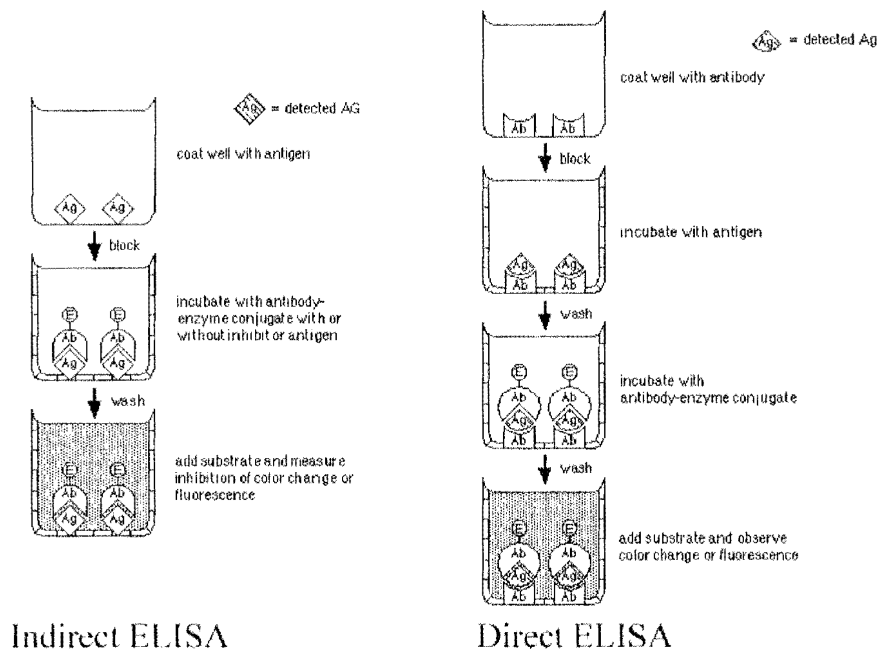


Figure 3.1: Schematic diagram representing the universally used indirect and direct ELISAs for antibody and Ag detection. This figure was taken directly from Coligan [12,14].

Critical parameters of the ELISA include its sensitivity, which depends on the antibodies used. Impurities found in most Ags can bind non-specifically to immunoglobulin produced from autoimmune responses [15]. Also, naturally circulating antibodies in a host could cross-react with the impurities or Ag itself, resulting in false absorbance readings. There is also the possibility, although infrequent, of excess binding in certain wells. The binding ability of most ELISA plates is due to irradiation of the polystyrene surface, thus any variation in the intensity could lead to non-specific wells which bind excess Ab or Ag [16]. An optimal ELISA uses Ab with high affinity and specificity in order to eliminate non-specific binding and cross-reactivity. Direct (sandwich) ELISAs are the most sensitive, detecting protein Ag concentrations between 100pg/ml to 1ng/ml while indirect ELISAs are an order of magnitude less sensitive.

3.2.1.2 Antibody-Antigen interaction

Antigen binds to an Ab within the variable region (Fig. 3.2). This antibody-antigen interaction is a dialogue between the Ab's binding site and the region on the Ag to which it binds, known as the epitope. Two different types of epitopes have been classified. The first type is a linear or continuous epitope where recognition is based primarily on the amino acid sequence with very little effect of conformation. The second type is conformational where the secondary, tertiary and quaternary structural elements of a protein bring together sometimes quite distant regions of the polypeptide chain. This type of binding is limited when proteins are unfolded.

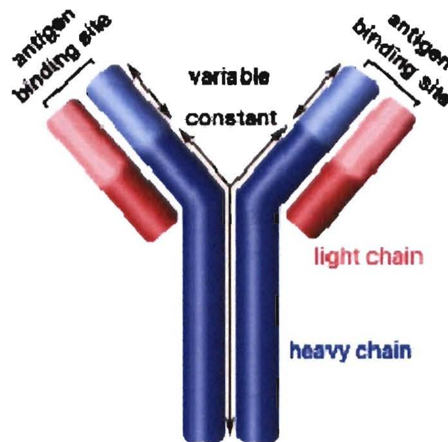


Figure 3.2: The Y-shaped monomer of an immunoglobulin molecule which consists of two identical heavy chains and two identical light chains. This figure was taken directly from http://www.bioscience.drexel.edu [17].

3.2.1.2.1 Stages involved during antibody binding

The initial binding is weak as Ab and Ag loosely interact by long range electrostatic interactions with diffusion limited orientation of the molecules (Fig. 3.3). They form an encounter complex held together by hydrophobic associations, which changes shape to form the actual Ab-Ag complex. Known as the lock-and key- mechanism the change in shape (docking) allows for an increase in the number of specific contacts (Fig. 3.3).

Structural studies have demonstrated that more specific antibodies have a more rigid (lock-like) binding site whereby the interactions with the epitope are dominated by electrostatic forces from salt bridges and hydrogen bonds [18]. In contrast, antibodies that are more cross-reactive have a more flexible binding site with fewer specific contacts and binding interactions are more hydrophobic in nature. Cross-reactivity with Ab that has a rigid binding site is also possible however efficient binding would require Ag with a high degree of molecular epitope mimicry (be it in amino acid sequences or three dimensional structures).

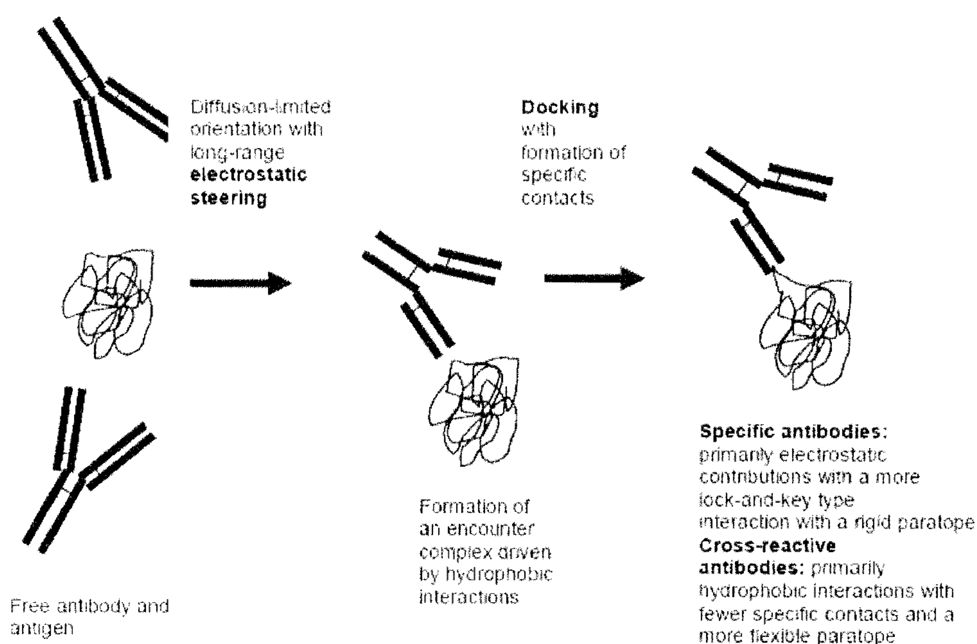


Figure 3.3: Schematic diagram of the stages involved in the association of an antibody with an antigen. This figure was taken directly from Breiteneder et al. [19,20].

3.2.1.3 Cross-reactivity

Cross-reactivity is the process by which Ab produced against one Ag binds to another Ag with common or structurally similar epitopes.

3.2.1.3.1 Cross-reactivity of helminths

Cross-reactivity of helminth Ags between taxonomically related [21] and unrelated [22] species as well as with allergens have become a concern in research and diagnostic analysis. The interrelationship between pathogens inside 1 host can influence the course of infection elicited by either parasite [23]. This has been demonstrated with *H. polygyrus*, which adversely affects the immune response to *T. muris* [24], *Hymenolepis citelli* [25] and *T. spiralis* [26]. Although the basis of this immunological interference is not fully understood, a possible explanation is the cross-reactivity of antibodies between species. *H. polygyrus* induced Ab was shown to cross-react with *T. muris* muscle larval Ag [22]. Similarly, sera from *S. obvelata* infected mice are known to cross-react to related species, *S. mesocriceti* (hamster pinworm) and *S. muris* (rat pinworm) as well as produce significantly high Ab titers to non-parasitic antigenic stimuli [27]. This suggests that *S. obvelata* might modulate the immune system of the host and therefore affect experimental results [27].

3.2.1.4 Polyclonal antibodies

Antigens in general are complex structural molecules that possess several epitopes which are recognized by specific receptors on lymphocytes [13]. Thus sensitization with any Ag, even highly purified will lead to the stimulation of several clones, each recognizing a different or partially identical epitope on the same Ag consequently producing polyclonal Abs. Polyclonal Abs are easily generated by sensitizing animals with an Ag plus an adjuvant to induce large quantities of Ab against the Ag. The antiserum is then tested by ELISA to obtain a high titer, which is collected and stored. These Abs are heterogeneous with respect to specificity and immunoglobulin (Ig) class of single Abs [13].

3.2.1.4.1 The advantage and application of polyclonal antibodies

Generating polyclonal Abs is less time consuming with minimum effort from a variety of animals. They require simple and readily available equipment and are particularly useful for analysis of denatured protein forms in immunoprecipitation, immunoblotting and ELISAs. In addition, polyclonal Abs can be raised against synthetic peptides if conjugated to appropriate carrier molecules [12].

3.2.1.5 Generation of monoclonal antibodies (hybridomas)

The limitation of antiserum as a source of Abs (polyclonal) and obtaining specific monoclonal Abs was overcome in 1975 when Köhler and Milstein [28] succeeded in demonstrating that, when fused with plasma cells from sensitized donors, plasmocytoma cells growing *in vitro* secreted the specific Ab of the plasma cell in addition to its own [13]. The specific Ab producing hybrid cells derived after fusion of a myeloma (cancerous plasma cell) with a plasma cell are called hybridomas. Myeloma cells can readily be cultivated and mutant myeloma cell lines incapable of producing Abs are used. As illustrated in Figure 3.4, animals are sensitized with an Ag plus an adjuvant until a large quantity of Abs is produced. The spleen is removed and teased into a single cell suspension which is fused with myeloma cells. The ratio of spleen to myeloma cells is largely dependent upon the animal used. In addition, polyethylene glycol (PEG) is added to the fusion to promote membrane fusion [11]. The fusion mixture is then transferred to a selective medium containing a combination of hypoxanthine, aminopterin, and thymidine (HAT). Aminopterin, a folic acid antagonist, is a poison that blocks the main pathway for the synthesis of pyrimidine and purine in cells [11,13]. Myeloma cells therefore die as they lack the enzyme that allows their growth in the presence of aminopterin. However spleen cells by-pass the pathway required by using the intermediated metabolites provided, hypoxanthine and thymidine. Unfused spleen cells die naturally within a week or 2, resulting in the survival of only the fused hybridomas. Hybridomas having Ab producing capacity as the original spleen cell are tested for the desired Ab and if positive, the cells

are further sub-cloned. The clone is immortal and produces monoclonal Ab of a single specificity [11].

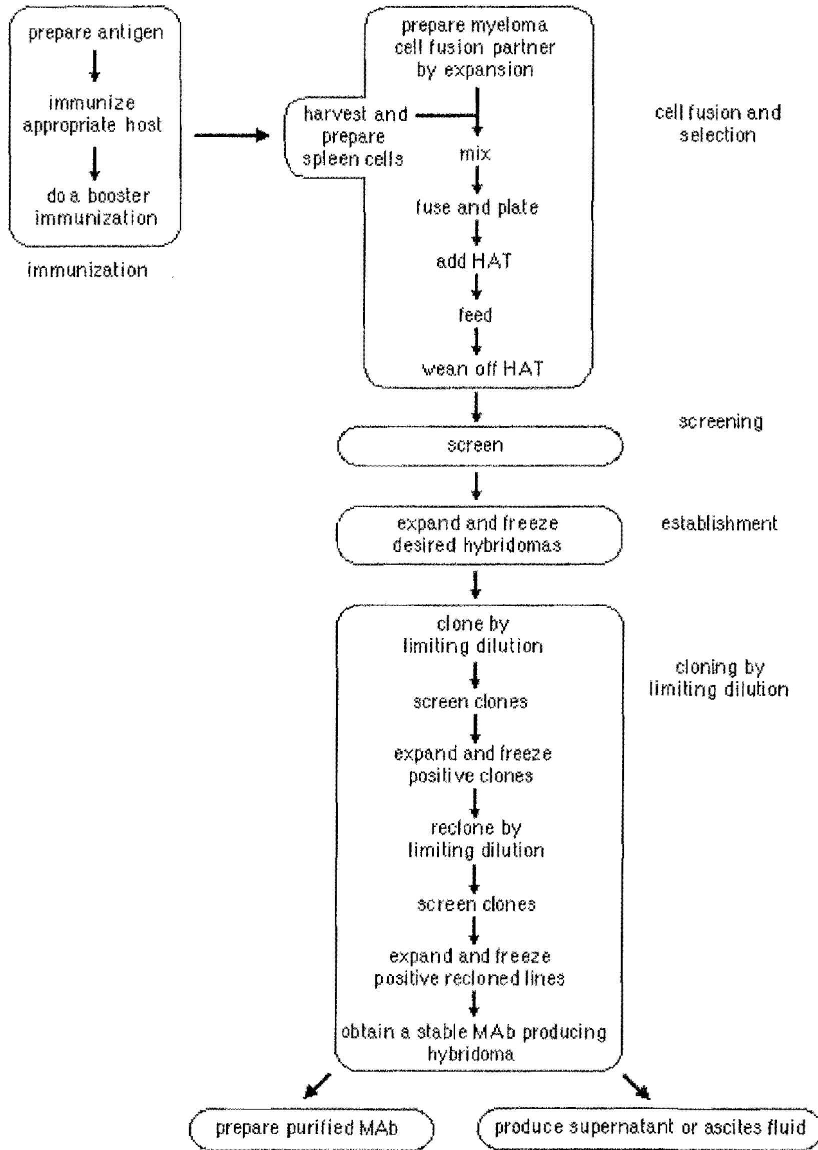


Figure 3.4: Stages of monoclonal Ab production. This figure was taken directly from Coligan et al [12].

3.2.1.5.1 Application of monoclonal antibodies

Monoclonal Abs within a short time has become an invaluable tool in many applications including their usage in tissue typing, identification and epidemiological study of infectious microorganism, identification of tumor and other surface Ags, classification of leukemias, and the identification of functional populations of different types of T cells. Anticipated future uses some of which are already being implemented include; passive immunization against infectious agents and toxic drugs, tissue and organ graft protection, stimulation of tumor rejection and elimination, manipulation of immune response, preparation of more specific and sensitive diagnostic procedures, and delivery of antitumor agents (immunotoxins) to tumor cells [11].

3.2.2 Hypothesis

Sato et al. were the first to show a humoral immune response to *Syphacia obvelata*. To optimize the detection of antibody and establish a *Syphacia obvelata* surveillance protocol we aimed to isolate and purify pinworm antigen and generate specific monoclonal and polyclonal antibody for the early detection and prevention of a pinworm outbreak.

3.3 Material and Methods

3.3.1 Animals

Mice, wistar rats and a Swiss hare were obtained from the Ysterplaat farm in the Western Cape, South Africa and maintained in the animal facility at the University of Cape Town. Animals were used for experiments performed in accordance with the guidelines of the Animal Ethics Research Board of UCT (Cape Town, South Africa).

3.3.2 Methods of isolating *S. obvelata* worms and eggs

3.3.2.1 Cellophane (Scotch tape) method [2]

Adhesive tape was applied to the perianal region of the mouse. Eggs were freed and collected by washing the adhesive surface of the tape with either water or saline.

3.3.2.2 Lewis and D'Silva's method [3]

Using a rice smear technique to collect eggs from the perianal area, rice grains were washed and boiled until the colcoptile areas were soft. The boiling water was drained and the rice, following overnight cooling, was smeared onto a glass slide, which was applied to the mouse's perianal region. Eggs lodged in the rice smear were examined under a light microscope to determine the yield.

3.3.2.3 Stahl's method [4]

The cecum of susceptible mouse strains (IL-$13^{-/-}$, IL-4/$13^{-/-}$, IL-4R$\alpha^{-/-}$) were collected in 0.65% NaCl (Merck, SA) and incubated in a gauze mesh at the mouth of a conical flask for 1-2hrs at 37°C. The worms were collected at the bottom of the flask and washed 3 times in 0.65% NaCl (1500rpm for 5min) before being processed for either eggs or Ag.

3.3.3 Producing an arbitrary standard

BALB/c wild type mice (4-5mice/group) were sensitized intraperitoneally (i.p.) with 50μg/ml Ag (*S. obvelata* somatic Ag) plus Complete Freund's Adjuvant (CFA). After 21 days the mice were administered a booster at 50μg/ml plus Incomplete Freund's adjuvant (IFA). The mice were euthanized 35 days post initial sensitization, their serum pooled and tested for Ag-specific IgG1 and IgG2b antibodies respectively. The serum samples were diluted 1/100 and used as an arbitrary standard for Ag specific ELISAs.

3.3.4 ELISA

All ELISAs were performed as previously describe in chapter 1 materials and methods.

3.3.5 Mycoplasma detection ELISA

Mycoplasma is a common contaminant of cell cultures affecting the reliability of biological research involved with cultured cells. Its origin can stem from bovine serum, laboratory personnel and other already infected cultures. Studies have shown that cell cultures are frequently contaminated with up to 30% [29-32] mycoplasma. The organisms induce a variety of effects on the infected cells in culture such as changes in metabolism, growth, viability and morphology. However, these alterations are not always that easily

visible and many mycoplasma contaminants grow slowly and do not destroy host cells but do affect the above mentioned parameters.

To detect for mycoplasma, 96-well microtitre plates were coated with 240μl/well of a mixture (60μl each) of 4 species (*Mycoplasma arginini*, *M. hyorhinis*, *Acholeplasma laidlawii* and *M. orale*) of anti-mycoplasma Ab (Roche, Germany) for 2hrs at 37°C. Plates were then blocked with 250μl/well blocking solution and incubated for 30min at 37°C. Appropriately diluted sample supernatant was then added and incubated O/N at 4°C. A positive (provided with kit) and negative (sterile media) control was also included. Following washing, 240μl/well of a mixture (60μl each) of the 4 species of anti-mycoplasma biotinyalyted secondary Ab (Roche, Germany) was added and incubated for 2hrs at 37°C. The plates were again washed and samples developed with 200μl/well streptavidin alkaline phosphatase Ab incubated for 1hr at 37°C with 200μl/well alkaline phosphate substrate incubated for 30min at RT. The results were visualized at an absorbance of 405nm. Mycoplasma negative was colorless.

3.3.6 Rabbit polyclonal antibody

3.3.6.1 Bleeding from the auricular artery

After restraining the rabbit, fur around the central auricular artery was removed using a disposable scalpel (Aesculap, Germany). The exposed area was swabbed with 70% ethanol (Merck, SA). Tension was then applied to the ear, so as to insert a 21-G needle, attached to a butterfly infusion syringe set (Nipro, USA). The needle was inserted at an angel parallel to the artery, ~10mm into the arterial lumen, directed towards the base of the ear. Gentle aspiration with the syringe allowed for the desired volume to be collected in a serum separating tube. The needle was withdrawn and digital pressure applied with a gauze sponge until bleeding stopped [12].

3.3.6.2 Protocol for producing polyclonal antibody

Pre-bled serum samples were obtained from rabbits prior to sensitization, to be used as a naïve Ab control for polyclonal Ab analysis. In a 10ml Greiner tube (Greiner bio-one, Cellstar, Germany) a volume of 1ml vortexed CFA (dispersed insoluble *Mycobacterium tuberculosis* bacilli) was added to 1ml of 240μg/ml *S. obvelata* Ag in dH_2O. The CFA/Ag mixture was vortexed vigorously until a homogeneous, white emulsion was formed. The stability, of the emulsion was tested on the surface of cold water, as its ability to hold together as a good oil-in-water emulsion. The rabbits were restrained and injected with 2ml adjuvant/Ag emulsion into 6 subcutaneous sites on their backs (~333μl/site). The first sensitized blood serum samples were obtained 14 days after the primary sensitization. Three weeks following, the first booster was administered (Ag prepared in IFA). The rabbits were bled 14 days thereafter (Fig. 3.10A). The second booster sensitization was administered 42 days following the primary sensitization, (again, Ag prepared in IFA). The rabbits were again bled 14 days thereafter. The rabbits were continually bled weekly for polyclonal Abs (Fig. 3.10A).

3.3.6.3 Quantifying the rabbit polyclonal antibody titer

The 96-well microtitre ELISA plates were coated with 5μg/ml *S. obvelata* Ag (Ag/PBS) and incubated for 3hrs at 37°C and/or O/N at 4°C. Plates were then blocked with 2% milk powder at 37°C for 3hrs and/or O/N at 4°C. This was followed by the addition of serum samples with doubling dilutions starting with 1/1000 and ending with 1/256000 (9 dilutions). Serum samples were incubated for 1.5hrs at RT. Plates were developed with goat anti-rabbit isotype-specific polyclonal alkaline phosphatase labeled Abs (1/5000 IgG) (Southern Biotechnology Associates, Inc), incubated for 1hr at RT, followed by the addition of 50μl/well of alkaline phosphate substrate. The absorbance was read between 405 and 492nm at 15min intervals by a Versamax turnable microplate reader.

3.3.7 Rat monoclonal antibody

Rats were sensitized using the same procedure as for polyclonal Ab (Fig. 3.11A). When the titer of the desired Ab was high enough the rats were killed, their spleens aseptically removed and prepared for fusion.

3.3.7.1 Feeder layer

A naive Wistar rat was killed by carbon dioxide and its spleen aseptically removed. The isolated spleen was teased through a 70μm nylon cell strainer and the cell suspension pelleted at 1200rpm for 5min. The pellet was resuspended in 5ml Red Cell Lying Buffer (RCLB), incubated for 5min at RT and underlayered with 2ml FCS. The cells were spun down at 1200rpm for 5min followed by two washes in 40ml DMEM-0 at 1200rpm for 5min. Finally the cells were concentrated at 1×10^6/ml and 100μl/well dispensed into a 96-well plate. Plates were incubated at 37°C in 5%CO_2 until the day of fusion (~ 1 week).

3.3.7.2 Fusion

Three days before fusion the rat showing the highest specific Ab titer (indirect ELISA) was administered a boost with 10μg of Ag (i.p) resuspended in 200ml of sterile PBS. On the day, the rat was killed, its spleen aseptically removed and placed in ~30ml of ice-cold DMEM/10%FCS (DMEM-10). Single cell suspensions were prepared as described for the feeder layer except that DMEM-10 was used. After the final wash, the cells were diluted and counted on a haemocytometer to determine the cell concentration and viability (10% trypan blue).

3.3.7.3 Preparing cells

While spleen cells were washed, myeloma cells (SP2) were separately harvested at 1500rpm for 5min. Myeloma cells were pooled and resuspended in DMEM before being washed thrice in 50ml DMEM (1500rpm, 5min). Separately spleen and myeloma cells were resuspended in 10ml complete serum-free DMEM (DMEM-0). The cells were counted and their viability checked (100% viability was recommended). The spleen and myeloma cells were mixed at a 1/1 ratio to a total volume 50ml DMEM-0. The suspension was pelleted, then aspirated, to which 1ml pre-warmed 50% PEG was sterilely added. One drop over a minute was added with gentle stirring. The fused cell suspension was gently mixed for another minute before the addition of 2ml DMEM-0 at a rate of 1ml/min with gentle stirring. This was followed by the addition of 7ml pre-warmed complete DMEM-0 over 2-3min (macroscopic clumps of cells were visible at this point). The suspension was centrifuged at 1500rpm for 5min, the supernatant discarded and the fused cells resuspended in 50ml complete DMEM-20/HEPES/pyruvate. Finally the cells were washed twice (1500, 5min) before being plated at 100μl/well into a flat bottom 96-well plate at a concentration of 2.5×10^6 total cells/ml. Plates were incubated at 37°C in 5% CO_2.

3.3.7.4 Monitor and feed cells

After 1 day of incubation the cells were checked for obvious clumping and a nearly confluent monolayer of viable cells on the bottom. A volume of 100μl complete DMEM-20/HEPES/pyruvate/ HAT per well was added. Cells were then monitored on consecutive days for confluent growth, half the volume aspirated and an addition of 100μl/well DMEM-20/HEPES/pyruvate/ HAT added. On day 14, half the volume was aspirated and 100μl complete DMEM-20/HEPES/pyruvate/HT added. Plates were incubated at 37°C in 5% CO_2. On day 15 and subsequent, the cells were feed DMEM-20/HEPES/pyruvate without HAT or HT.

3.3.7.5 Screening primary hybridoma supernatants

Growing hybridomas were identified using an inverted microscope (Nikon) and allowed to grow (37°C in 5%CO_2) without feeding for ±2 days (saturated Ab titer). A volume of 100μl was assayed by ELISA to identify positive hybridomas secreting anti-*Syphacia obvelata* Ab. Positive samples were transferred to a new 96-well plate and cells fed complete DMEM-20/HEPES/pyruvate.

3.3.7.6 Establishment of hybridoma lines

Confluent hybridomas were expanded to 24-well plates. The initial clones (96-well master plate) were resuspended in 150μl complete DMEM-20/HEPES/pyruvate and incubated at 37°C in 5%CO_2. To the 24-well clones 1ml of complete DMEM-20/HEPES/pyruvate was added and the plates incubated for 2-3 days (37°C in 5%CO_2). After the cells were 25% to 50% confluent they were cloned by limiting dilution. The remaining hybridomas were pelleted (1500rpm for 5min), frozen and stored at -80°C/liquid nitrogen.

3.3.7.7 Cloning by limiting dilution

Candidate hybridomas identified by ELISA were further cloned by limiting dilution to obtain a single cell producing only a single Ab (monoclonal antibody).

Candidate hybridoma lines were counted and their viability assessed using a haemocytometer. Ten milliliters of cells at 50 viable cells/ml and 10ml at 5 viable cells/ml were prepared in complete DMEM-20/HEPES/pyruvate. The cells were plated in a 96-well plate at 200μl/well and incubated for 7 to 10 days in a humidified incubator (37°C in 5%CO_2). Wells with optimal monoclonal growth were inspected with an inverted microscope and monoclonality identified by tight single clusters of cells. More than one cluster of cells possibly represents polyclonal growth. Supernatant from singly

clustered wells were screened by ELISA using supernatant from the original hybridoma as a positive control. The desired clones identified were expanded, frozen and stored at -80°C/liquid nitrogen. The hybridomas suspected of producing monoclonal antibody (MAb) were plated a second time in a 96-well at 0.3 cells/well (60 viable cells in 40ml media). The previous steps were then repeated with the resulting monoclonal growth inspected for single clusters, assayed by ELISA, expanded, frozen and stored. Positive monoclonal hybridomas were weaned to complete DMEM-10/HEPES/pyruvate to establish a stable cell line.

3.3.8 Growing hybridomas for purification

Samples thawed on ice were added to 25ml DMEM. The suspension was then centrifuged at 1200rpm for 10min. The supernatant discarded, and pellet resuspended in 1.5ml media. Suspended hybridoma cell lines were serially diluted in 24-well plates and incubated at 37°C in 5%CO_2. After proliferating cells reached confluency (turns yellow) they were transferred to a small flask then medium flask and finally a triple flask. Large volumes of supernatant containing the Ab of choice were pooled for purification.

3.3.8.1 Purifying antibodies

Protein G is a bacterial cell wall protein. It is isolated from group G streptococci and binds most mammalian immunoglobulins through their Fc regions [33]. It is this unique property of the protein that is used in affinity chromatography to purify mammalian monoclonal and polyclonal antibodies [33].

3.3.8.1.1 Preparing the protein G-column

Before preparing the protein-G sepharose (Amersham Pharmacia, Sweden) the glass column apparatus was washed (dH_2O, 70% ethanol, dH_2O) and gas sterilized. The column was then vertically attached to a stand and plugged at the bottom. Sepharose

solution containing 20% ethanol was slowly dispensed along the side of the chamber using a glass pipette to eliminate any bubbles from forming. The gel was allowed to settle, the bottom unplugged to drain the fluid and allow further settling of the sepharose gel. Finally the column was washed with 20% ethanol, followed by 150ml 1×PBS before being standardized to run at 1ml/minute.

3.3.8.1.2 Purifying and selecting IgG isotype antibody on a protein-G Sepharose column

One liter of the supernatant containing the Ab was run at 4°C O/N. The column was then washed with 150ml 1×PBS and the Ab eluted with 30ml of 0.1M Citric acid (pH 2.4) into 30 eppendorf tubes containing 50μl of 2M Tris buffer. The aliquots were mixed and their concentrations calculated (Absorbance at 280nm × dilution factor/IgG factor, 1.38). The aliquots with the highest Ab titers were pooled, dialyzed (>12000 Mw, Sigma), filtered (0.2μm, Millipore) and finally concentrated for storage (-80°C).

The column was rinsed with 150ml 1×PBS followed by 20% ethanol.

3.3.9. Denaturing (SDS) discontinuous gel electrophoresis

One-dimensional gel electrophoresis under denaturing conditions (i.e. in the presence of 0.1% SDS) separates proteins based on the molecular size as they move through a polyacrylamide gel matrix within an electric field. The strong negative polarity of sodium dodecyl sulfate (SDS) allows all the sample proteins to be approximately equal in negative charge. The polyacylamide gel consists of a separating gel topped with a stacking and secured in an electrophoresis apparatus. After sample proteins are solubilized by boiling (to linearize protein structure) in the presence of SDS, an aliquot of the protein solution is applied to a gel lane, and the individual proteins are separated electrophoretically. A molecular weight marker electrophoresed with the protein solution allows the determination of the various protein weights within the sample protein profile.

3.3.9.1 Separating gel

The two glass plates and two 0.75mm spacers of the electrophoresis (sandwich) apparatus (Bio-Rad, USA) were cleaned with 70% ethanol before being assembled. In a 50ml Greiner tube an 11% separating gel (9.9ml 30% acrylamide/0.8%bisacrylamide, 6.75ml 4× Tris·Cl/SDS, pH8.8, 125μl 10%w/v AMPS, 10μl TEMED and 10.35ml dH_2O) was prepared and poured ¾ to the top of the assembled sandwich apparatus. The remaining space was topped with H_2O-saturated isobutyl alcohol or 2-propanol (Merck, SA) to ensure a uniform polymerization of the separating gel (RT for an hour).

3.3.9.2 Stacking gel

The H_2O-saturated isobutyl alcohol (2-propanol) was poured off the polymerized separating gel, which was then rinsed with 1×Tris·Cl/SDS pH8.8 buffer (residual isobutyl alcohol can reduce resolution of the protein bands). In a 15ml Greiner tube a stacking gel was prepared (1.4ml 30% acrylamide/ 0.8%bisacrylamide, 2.0ml stacking gel, 25μl 10%w/v AMPS, 50μl TEMED and 4.6ml dH_2O) and poured to about 1cm from the top of the assembled sandwich apparatus. This was followed by the insertion of a 0.75mm Teflon comb into the layer of stacking gel solution. The solution was allowed to polymerize at RT for an hour.

3.3.9.3 Sample preparation

Samples at an appropriate concentration and mixed with loading dye were boiled to remove endogenous proteases that are very active in the loading buffer prior to boiling. Also the tertiary and quarterary protein structures linearize allowing the highly negative SDS molecules to bind and ensure separation by molecular weight only.

3.3.9.4 Sample loading and gel electrophoresis

The comb was carefully removed and lanes washed with 1×SDS running buffer. The gel sandwich apparatus was attached to the upper buffer chamber and placed in the 1×SDS running buffer filled lower buffer chamber. The upper buffer chamber was partially filled with 1×SDS running buffer prior to loading the 10-50μl protein sample aliquots. The upper chamber was then completely filled with running buffer and electorphoresed at 20mA for 4-5hrs (large gel) or 400mA for 1hr (small gel) under cooling.

Protein sizes (kDa) were calculated by comparison to the rate of migration of a recombinant protein molecular weight marker (Rainbow marker, Bio-Rad, USA). A volume of 5-10μl rainbow molecular marker was loaded per reaction (appendix B).

3.3.10 Immunoblotting and immunodetection (Western blot)

3.3.10.1 Assembling of the immunoblot sandwich

On completion of electrophoresis, the gel sandwich was disassembled, the stacking gel was removed and the separating gel trimmed (removed excess gel). This was followed by the assembly of the horizontal transfer sandwich cassette, placed sponge, followed by a sheet of filter paper cut to the same size as the gel, and pre-wet with transfer buffer (3.02g/L Tris, 14.4g/L glycine, 20% methanol) onto the anode inside the transfer tank. The nitrocellulose transfer membrane (Hybond-ECL, Amersham, Bioscience, UK) was placed onto the filter paper and air bubbles expelled. The surface of the gel was moistened with transfer buffer and placed onto the nitrocellulose membrane followed by the removal of air bubbles still remaining. Another piece of filter paper was added, then sponge, followed by the cathode to complete the horizontal transfer sandwich cassette assembly. The sandwich was placed into a transfer tank filled with transfer buffer. Proteins were electrophoretically transferred from gel to nitrocellulose membrane O/N at 20mA (large) or 1hr at 400mA (small), in a cold room or on ice (4°C).

3.3.10.2 Visualization of *S. obvelata* protein

Following the completion of the electophoretic protein transfer, the nitrocellulose blot was trimmed and then blocked in 2% milk powder at RT for 1hr with constant agitation on a rocking platform (Gyro-rocker-STR 9, Stuart, UK). The blot was washed 5 times over a 25min span (5min washing intervals), followed by the addition of 1/1000 primary Ab solution (anti-*S.obvelata* antibody). After a 2hr incubation at RT on a rocker the blot was washed as before and 1/1000 goat anti-Ig (anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, IgA, IgE) secondary alkaline phosphatase Ab added. The 2nd Ab was incubated for 1-2hr at RT on a rocker, the blot was washed and the protein visualized by the chromogenic substrate, BCIP/NBT (½ tablet in 15ml dH_2O per blot).

3.4 Results

3.4.1 Establishing an indirect pinworm specific ELISA

Although less sensitive than a sandwich ELISA, the indirect ELISA is easy and simple to establish. To evaluate the optimal coating concentration for detecting mouse anti-*S. obvelata* Ab we assayed pinworm infected serum against five Ag concentrations. Wells coated with 5μg/ml showed a 1000-fold increased binding sensitivity when compared to concentrations lower or higher respectively (Fig. 3.6A and B). Hence, 5μg/ml coating concentration was used as the standard for *S. obvelata* endpoint titrations.

3.4.2 Preparing hyper-immune serum

To simplify the quantification of anti-*S. obvelata* Ab, we generated hyper-immune serum to reference the Ab levels of infected mice. BALB/c mice were sensitized intraperitoneally using 50μg/ml plus CFA, as demonstrated in Fig. 3.7A. In response, BALB/c mice elicited elevated IgG1 (Fig. 3.7B) and IgG2b (Fig. 3.7C) isotype antibodies. Sera from infected mice were pooled, diluted 1/100 and used as a positive control and arbitrary standard with 5μg/ml coating Ag.

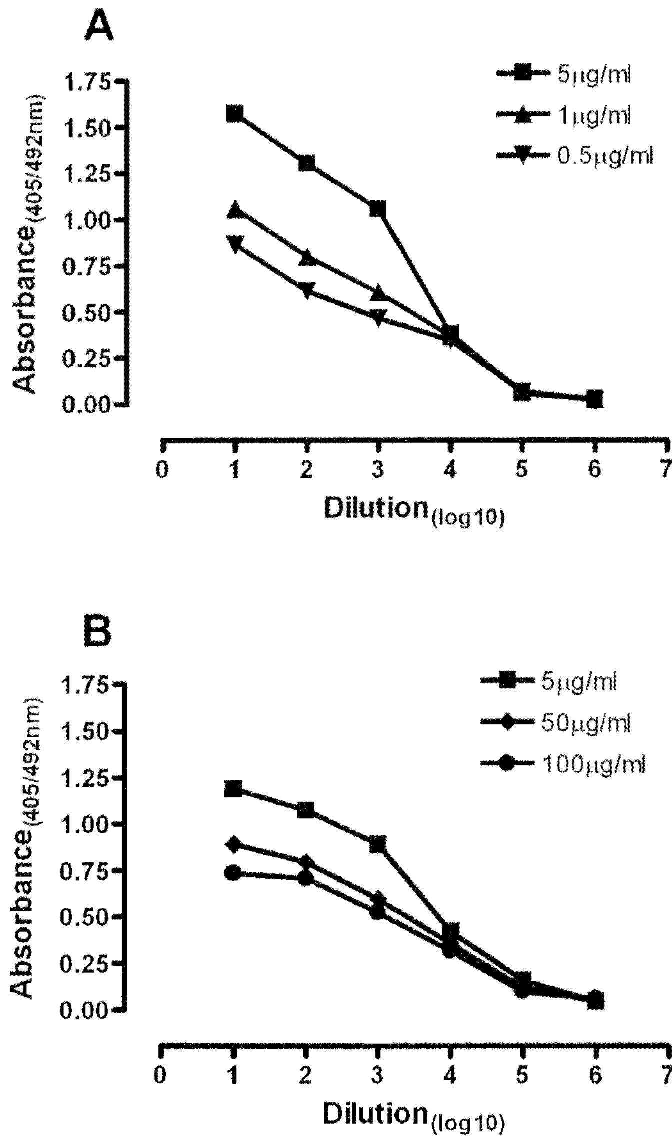


Figure 3.5: An end-point titration to determine a standard *S. obvelata* coating concentration. Soluble pinworm Ag was diluted to various **(A)** low (0.5, 1 and 5μg/ml) and **(B)** high concentrations (50 and 100μg/ml) and analyzed by an indirect ELISA for the optimal coating sensitivty against parasite-specific IgG2b. Pooled sera from 4-5 mice infected with *S. obvelata* were used.

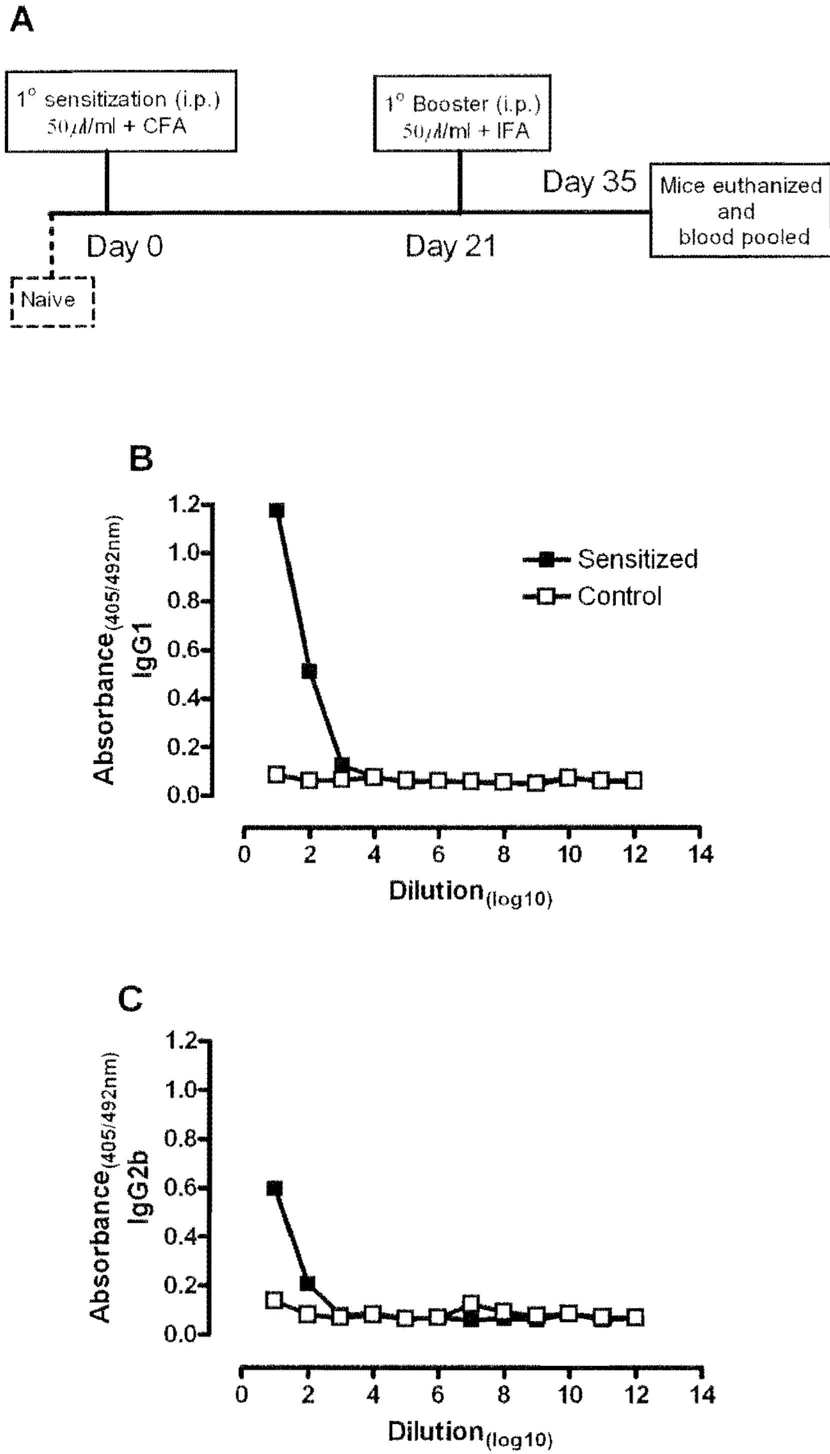


Figure 3.6: Anti-*S. obvelata* hyper-immune serum was generated in BALB/c mice for use as positive control and arbitrary standard. **(A)** Sensitization protocol for generating anti-*S. obvelata* IgG1 **(B)** and IgG2b **(C)** isotype Ab.

3.4.3 Analyzing the cross-reactivity of anti-*S. obvelata* serum against antigen of other research parasites

Adventitious infections skew immune responses and induce false experiment results. To rapidly and correctly identify *S. obvelata* for eradication we determine the specificity of mouse anti-*Syphacia obvelata* polyclonal serum (SOPS) by analyzing its cross-reactivity with Ag of commonly used research parasites and ovalbumin allergen. ELISA plates coated with 5μg/ml *Anisakis pegreffii*, *Ascaris lumbricoides*, *Trichuris muris* (somatic and excretory/secretory), *Nippostrongylus brasiliensis*, *Leishmania major*, *Schistosoma mansoni*, and Ova Ag respectively were assayed for specific-IgG1-and-IgG2b isotype Ab. Anti-SOPS antibody significantly cross-reacted with *A. pegreffii* (Fig. 3.8C) and *N. brasiliensis* (Fig. 3.8B) homogenates' showing elevated IgG isotype responses. This reaction was less detectable against *A. lumbricoides* (Fig. 3.8D), *T. muris* (somatic, Fig. 3.8E and excretory/secretory, Fig. 3.8F), *L. major* (Fig. 3.8G), *S. mansoni* (Fig. 3.8H) and Ova (Fig. 3.8I) homogenates with low Ab titers similar to naïve serum as measured by the indirect ELISA. The isotype Ab responses against *S. obvelata* Ag (5μg/ml) were used as control (Fig. 3.8A).

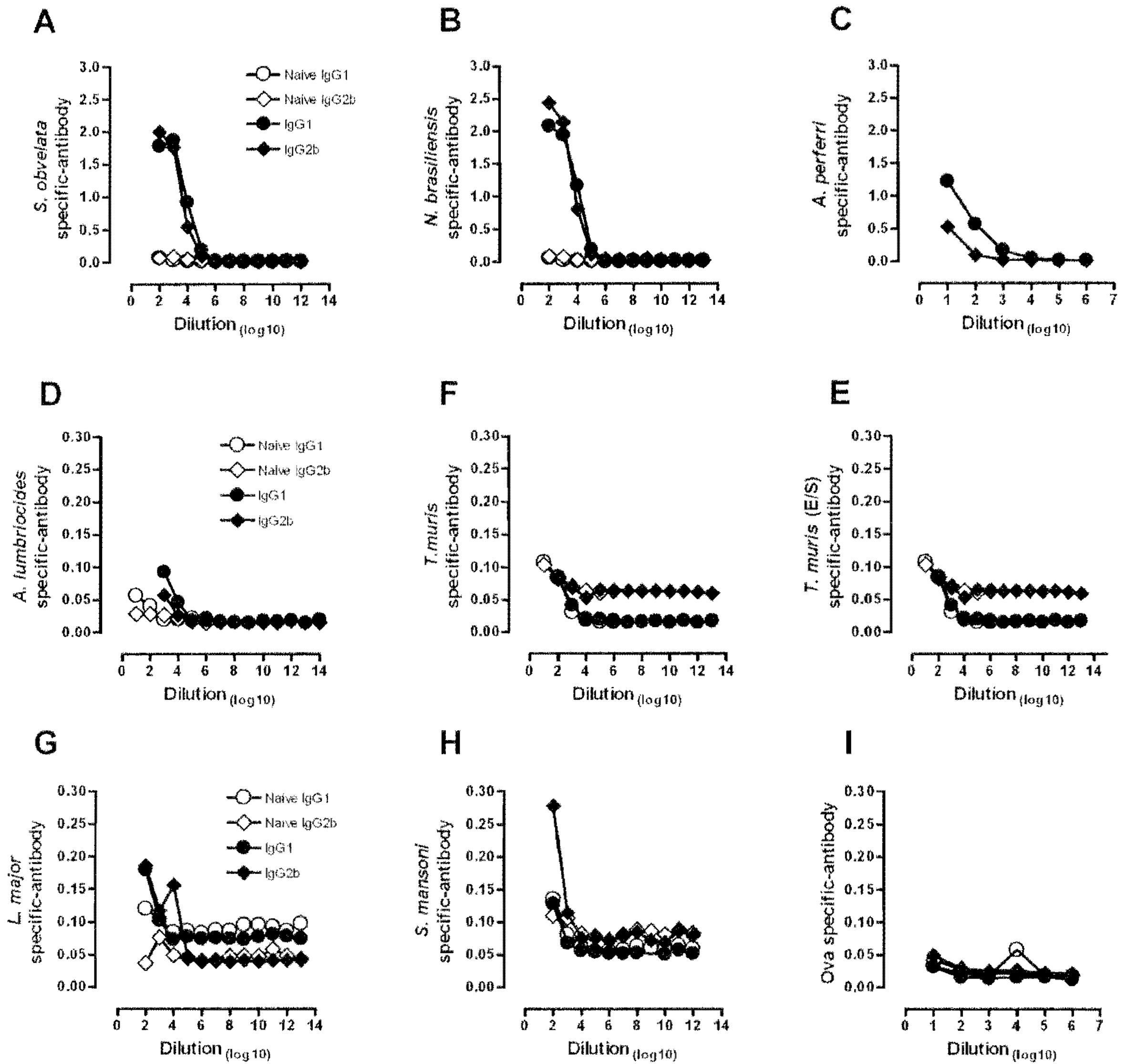


Figure 3.7: Cross-reactivity of anti-*S. obvelata* serum against Ag of other research parasites. Worm homogenates of *Syphacia obvelata* **(A)**, *N. brasiliensis* **(B)**, *A. pegreffii* **(C)**, *A. lumbricoides* **(D)**, *T. muris* somatic **(E)** and excretory/secretory **(F)**, , *L. major* **(G)**, *S. mansoni* **(H)** and ovalbumin **(I)**, were assayed against anti-*S. obvelata* sera in an indirect ELISA.

3.4.4 The efficacy of anti-*S. obvelata* polyclonal antibody in an ELISA

Sera from rabbits sensitized to *S. obvelata* antigen was collected weekly, pooled and assayed in an indirect ELISA. The concentration of the rabbit anti-*S. obvelata* polyclonal sera (SOPS) was not initially determined. Instead serial dilutions of 1/70, 1/140 and 1/700 were prepared in PBS and assayed in an end-point titration ELISA. Figure 3.10C, firstly demonstrates that the *S. obvelata* sensitization worked and that anti-SOPS recognized and bound *S. obvelata* antigen equally as efficient as mouse polyclonal Ab (Fig. 3.10B). Secondly, the figure shows that sera diluted 1/700 was sufficient to bind 5μg/ml of pinworm antigen. The sera from mammals are abundant in different proteins that are all specialized to perform different functions, including inhibition and/or degradation (proteases) of other proteins. As a result, proteases abundant at the higher concentrations of our 1/70 and 1/140 diluted anti-pinworm serum either degraded or diluted the rabbit sera allowing the higher concentrations to bind 5μg/ml of pinworm antigen similarly to the lower concentration of 1/700 (Fig. 3.10C). Rabbit anti-SOPS in combination with 5μg/ml *S. obvelata* Ag reacted 10^4-fold more sensitive against anti-*S. obvelata* IgG1 (Fig. 3.10D) and IgG2b (Fig. 3.10E) than any lower concentration. Naïve mouse serum was used as control.

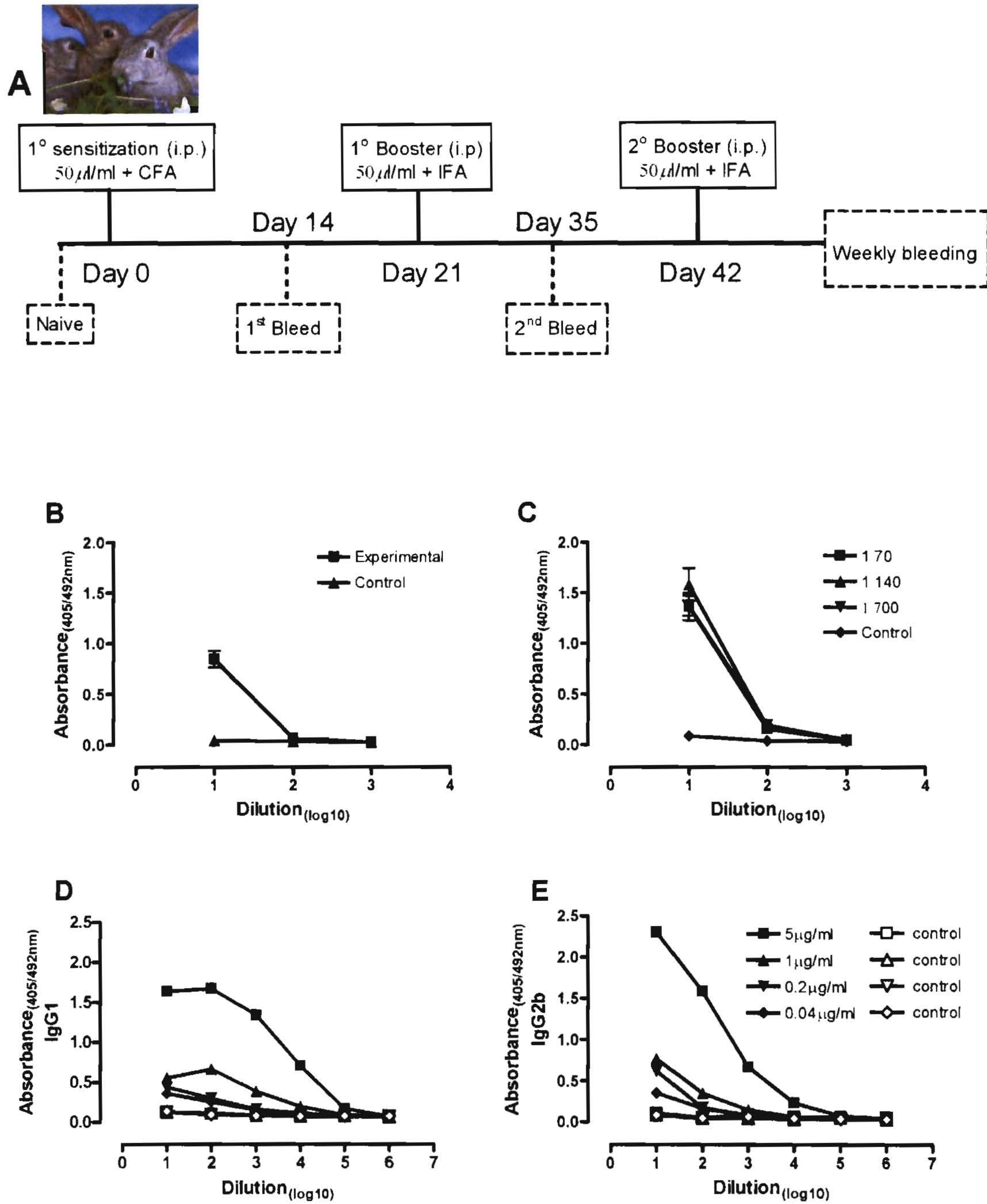


Figure 3.8: Rabbit anti-SOPS antibody is an effective diagnostic tool for detecting pinworm. **(A)** Sensitization protocol. An indirect ELISA showing the sensitivity of detecting pinworm Ag using mouse **(B)** and rabbit **(C)** anti-pinworm sera. A dilution of 1/700 rabbit anti-*S. obvelata* polyclonal Ab was sufficient in detecting 5μg/ml *S. obvelata* Ag. A dilution of 1/140 rabbit-anti-*S. obvelata* polyclonal Ab used as capture Ag in a sandwich ELISA proved significantly effective in detecting IgG1 **(D)** and IgG2b **(E)**. Naïve mouse sera were used as controls.

3.4.5 The efficacy of anti-*S.obvelata* monoclonal antibody in an ELISA

To increase the assay specificity we further modified our sandwich ELISA by introducing a rat anti-*S. obvelata* monoclonal coating Ab. Monoclonal antibodies produced were isolated, screened and purified before being analyzed for their binding sensitivity and specificity. Unfortunately, only one clone survived (4A4.3) producing MAb which reacted strongly against 5μg/ml *S. obvelata* Ag in an indirect ELISA (Fig. 3.11B). The monoclonal antibody also cross-reacted against 5μg/ml *T. muris*, *N. brasiliensis*, *L. major* and *S. mansoni* antigen (Fig. 3.11C). In addition, 4A4.3 as a capture Ab used in combination with parasite Ag in a sandwich ELISA proved unsuccessful as the monoclonal non-specific bound conjugated anti-mouse IgG antibody.

3.4.6 Antigenicity of *S. obvelata* somatic antigen

To identify the antigenic proteins of the *S. obvelata* somatic Ag to 6 Ab isotypes, hyper-immune sera (mouse anti-SOPS) from infected BALB/c mice were analyzed by immunoblotting. Immunoglobulin-(Ig)M (lane 1) the initial Ab response induced upon infection recognized 5 Ags of 51, 53, 54, 56 and 57 kDA respectively. These bands were also visualized with IgG (lane 2). Nevertheless, IgG and its subclasses (IgG1, lane 3; IgG2b, lane 4) strongly precipitated 4 indistinguishable proteins of 65, 64, 60 and 58 kDa. IgE, despite making up only a minute percentage of the Ab pool also reacted to the 4 high molecular weight proteins. Serum IgA (lane 6), showed no reaction to *S. obvelata* Ag. No bands were observed with control sera (data not shown).

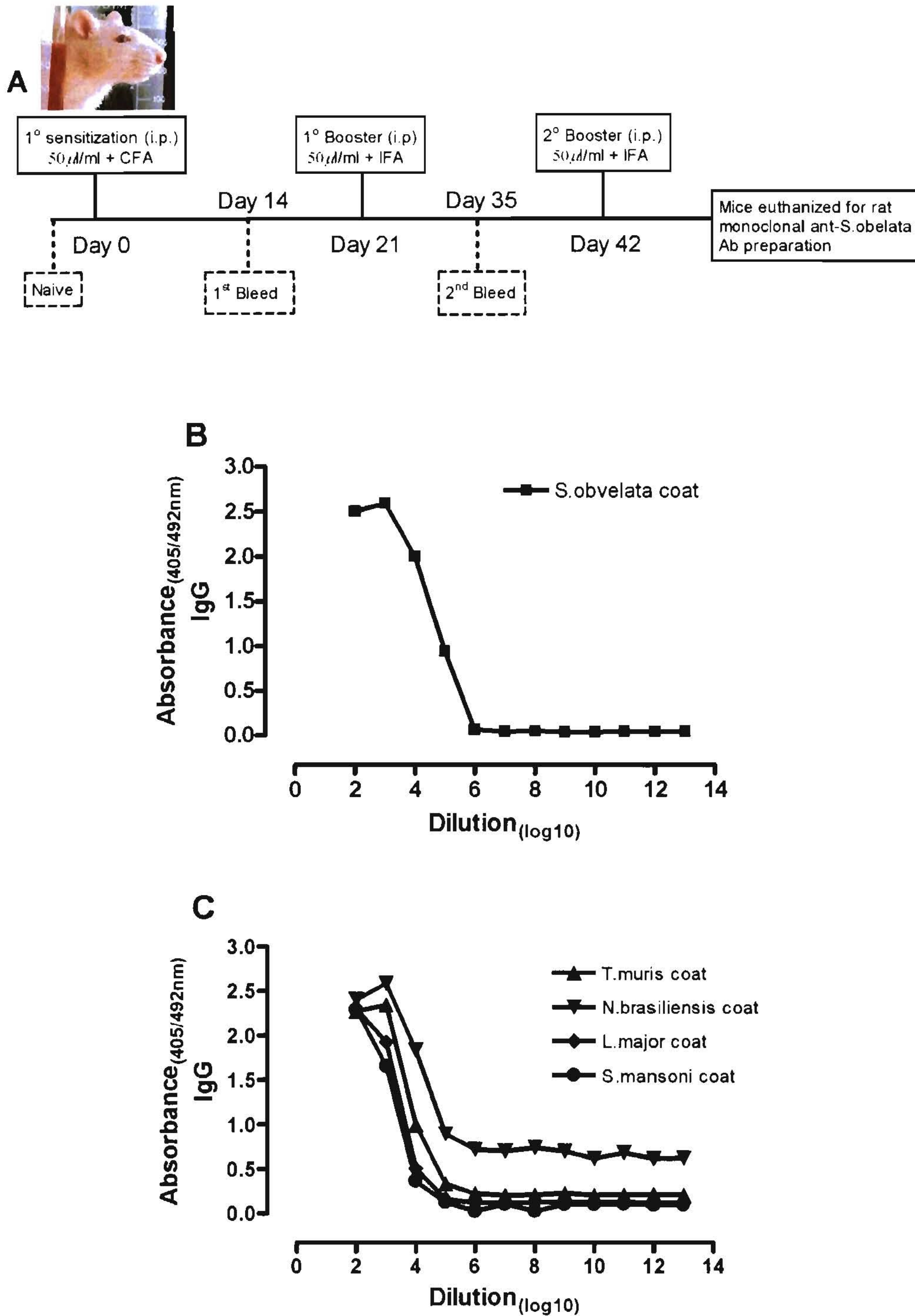


Figure 3.9: Production and analysis of monoclonal rat anti-*S. obvelata* antibody. **(A)** Sensitization protocol for generating rat anti-*S. obvelata* monoclonal Ab. **(B)** Reaction of 4A4.3 against *S. obvelata*. Ag **(C)** Cross-reactivity of 4A4.3 monoclonal antibody against 5µg/ml *T. muris*, *N. brasiliensis*, *L. major* and *S. mansoni* antigen in an indirect ELISA.

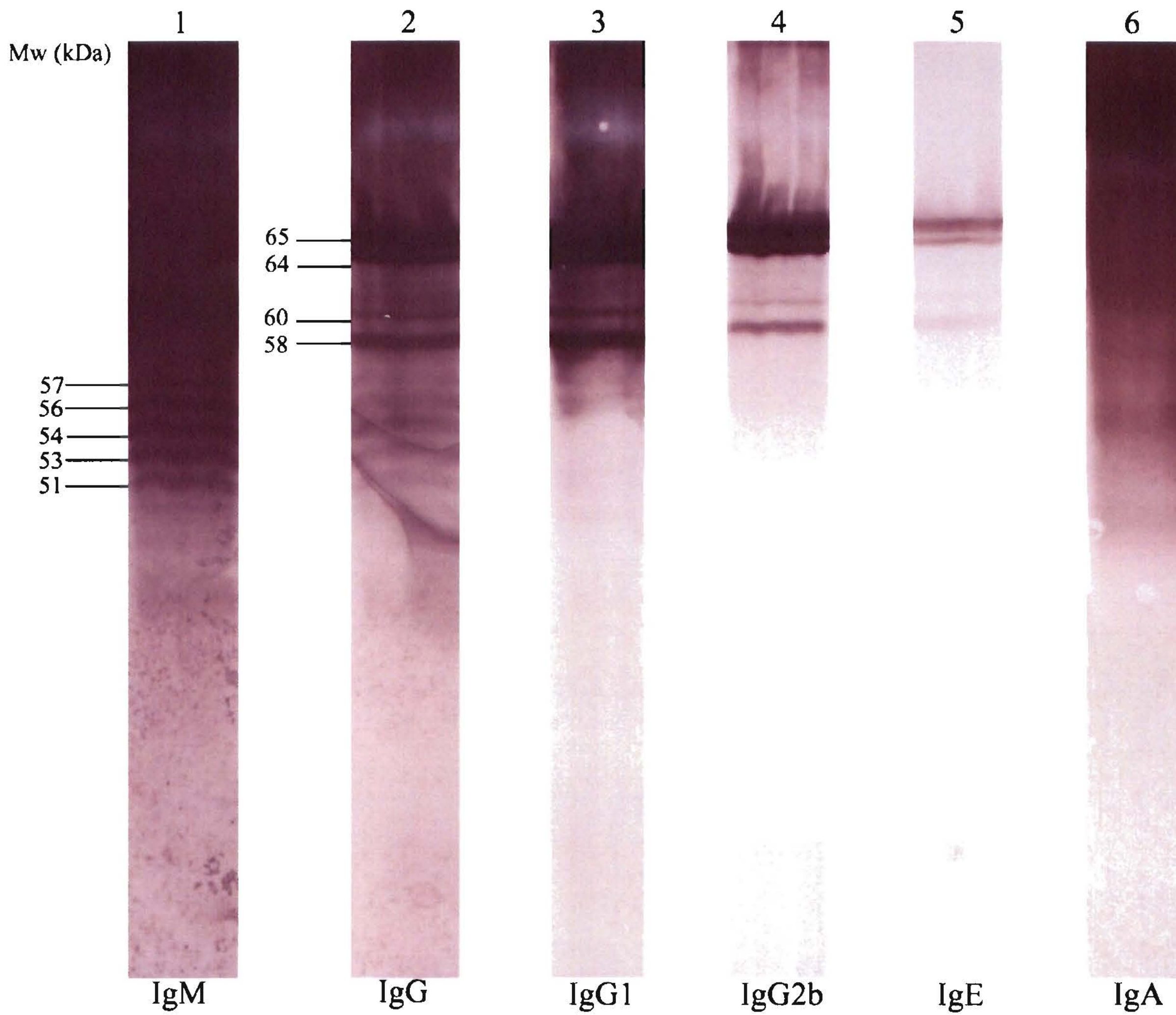


Figure 3.10: Antigenic protein profile of the Ags derived from *S. obvelata* in response to immunoglobulin isotypes. An amount of $10\mu g$ *S. obvelata* extract was separate electrophoretically on a 11% polyacrylamide gel. The separated proteins were transferred via an electric current to nitrocellulose paper before being bound by anti-pinworm isotype antibody and visualized by the chromogenic substrate, BCIP/NBT. **(1)**, IgM; **(2)**, IgG; **(3)**, IgG1; **(4)**, IgG2b; **(5)**, IgE; **(6)**, IgA. Mw, molecular weight

3.5 Discussion

Genetically engineered mice such as the cell-specific 'knock-outs' are the latest in the rapid advance of biotechnology and our quest as researchers in understanding and controlling the immune system. Their response to infection and disease can be unpredictable, adding to the already overwhelming problem of overcrowding and unwanted adventitious pathogens. It is important to emphasize the control and eradication of adventitious infections, as global exchange of animals and animal products increase among laboratories [1]. Although some of these parasites provoke overt disease, most cause subclinical infections that can, nonetheless, significantly alter research results [1].

In an approach to detect, control and eliminate *S. obvelata* infection we compared 3 isolation protocols. Using the cellophane tape [2] and Lewis and D'Silva's methods [3] we acquired low egg numbers and a mixture of *S. obvelata* and *Aspiculuris tetraptera* pinworm species. Stahl's method [4], by directly isolating worms from their site of maturation (cecum) provided a pure species population of *S. obvelata* and sufficient eggs for our infectious study. Although large numbers of infected mice were required using this method, and we cannot completely guarantee that a low *A. tetraptera* contamination did not occur, Stahl's method proved optimal.

Next we developed a pinworm-specific ELISA with an optimal coating concentration of 5μg/ml *S. obvelata* whole extract. Coating concentrations lower (1 and 5μg/ml) and higher (50 and 100μg/ml) in an end-point titration ELISA showed a decreased binding sensitivity to the optimal. The assay was standardized with an arbitrary standard of hyper-immune sera for quantifying Ab isotype responses in experimental infections. Although the hyper-immune sera (mouse anti-SOPS) had only been induced in BALB/c mice, the increased parasite-specific IgG isotype specificity allowed serological detection for all mouse strains. Previous studies by others have shown cross-reactivity of anti-*S. obvelata* Ab with *S. muris* (Wistar rat pinworm) and *S. mesocriceti* (golden hamster pinworm) but

not *Aspiculuris asiatica* (Mongolian gerbils pinworm) [27]. We report cross-reactivity with *A. pegreffii* and *N. brasiliensis* somatic Ag suggesting that common epitopes are shared by these GI nematode parasites. This was confirmed by immunoblotting (data not shown). The anti-*S. obvelata* polyclonal serum (SOPS) showed no reaction against parasite Ag of *A. lumbricoides*, *T. muris* (somatic and excretory/secretory), *L. major* and *S. mansoni* as well as to the food allergen, ovalbumin. However, with the exception of *S. obvelata*, the coating concentration of 5μg/ml *A. pegreffii*, *A. lumbricoides*, *T. muris* (somatic and excretory/secretory), *N. brasiliensis*, *L. major*, *S. mansoni*, and Ova Ag was not analyzed against various concentrations of anti-SOPS. As a result, the cross-reactivity of anti-SOPS may still be a limiting factor.

Limiting to the use of serology is the early detection of an Ab response, usually requiring one or more weeks after infection [16]. The delay can be devastating in an animal facility, as the rapid spread of *S. obvelata*, may alter experiments in mice concomitantly infected with the GI nematode [27,34]. To avoid such an outbreak, we increased our ELISA's sensitivity by developing rabbit anti-SOPS Ab. In a sandwich ELISA, capturing polyclonal Ab diluted 1/140 and used in combination with 5μg/ml *S. obvelata* Ag proved ideal in detected mice that had been exposed to pinworm. Comparatively, the direct assay reduced non-specific binding and showed an increased sensitivity allowing early detection of low concentrations of infected parasite serum. Rabbits were used to produce high Ab titers and reduce cross-reactivity (rabbit versus mouse Ab).

Continuing with the objective of developing a sustainable ELISA with the potential of becoming commercial we generated 66 successful monoclonal hybridomas against anti-*S. obvelata* somatic Ag. Unfortunately only one clone survived and the exact reason for all hybridoma death remains unknown. One possible explanation could be the inadequate preparation of hybridoma clones for storage (-80°C/liquid nitrogen). Nevertheless, MAb 4A4.3, an IgG isotype Ab reacted well against *S. obvelata* Ag in an indirect ELISA (Fig. 3.11B). The MAb however is a poor serodiagnostic tool albeit possible for detecting pinworm in SPF mice (indirect ELISA), the Ab cross-reacts with common research

parasite Ags (Fig. 3.11C) and cannot be used in a sandwich ELISA as it also cross-reacts with conjugated anti-mouse Ab.

Alternatively we identified individual Ags to improve our ELISA. Four dominant bands of 65, 64, 60 and 58 kDa were observed by immunoblotting. The high molecular weight proteins which reacted with resistant BALB/c polyclonal serum, suggest that these molecules are recognized by the host's immune system upon *S. obvelata* infection. Consequently, the development of monoclonal Ab against these molecules could provide better serodiagnostic candidates. The 65 kDa protein have previously been identified by others [27]. In their study, the authors visualized the antigenic protein 26 days postinfection as it reacted with *S. obvelata*-specific IgG. In our study, we reiterated their findings of Ab reactivity but not time (a parameter not investigated by us) and further demonstrated that the response extends to the IgG subclasses as well as IgE Ab.

From the described results, we have developed an improved ELISA system for detecting a pinworm infection and quantifying the induced Ab response. Used in combination with a sentinel program, which allow for the screening of small susceptible mouse groups, the anti-*S. obvelata* ELISA provides an effective method in preventing an outbreak. Moreover, we recognized favorably serodiagnostic candidate proteins for future analysis. Monoclonal antibodies generated against these targets for detecting pinworm will potentially increase sensitivity and specificity reducing the cross-reactivity of serum Ab.

It should be noted that further refinement to the ELISA could be achieved by designing a kinetic *S. obvelata* experiment in mice infected with different inoculums of eggs. In our study, we showed that mice infected with 500 pinworm eggs elicited a specific antibody response above a threshold absorbance of 0.1 for IgG1 and 0.2 for IgG2b as observed against serum from uninfected mice. Although we could detect the presence of *S. obvelata* antigen as early as 7 (IgG1 in BALB/c mice) and 14 (IgG2b in IL-4R$\alpha^{-/-}$ mice) days postinfection, a dose of 500 eggs is an exaggerated amount for the inoculum needed to infect mice. The advantage of using various egg inoculums in a kinetic would allow us to identify the earliest time point required to detect the presence of pinworm antigen by a

natural infection (low dose) most likely encountered by animals housed in a barrier facility.

Pinworm is a concern of both breeding and experimental animals housed in research facilities. Although animals are routinely tested for adventitious infections, the rapidity with which pinworm spread makes it an extremely dangerous parasite. This is emphasized in our study, as we demonstrated that the GI nematode induces a Th2-type immune response (Chapter I) and non-specifically influence the cytokine production in an allergy experiment (Chapter II).

Sentinel programs, which allow for the screening of small susceptible mouse groups, work well in detecting adventitious pathogens such as bacteria, viruses, protozoa and helminths (pinworm). Tests are done regularly but with a 3month or 6month interval which might be too late for early detection of pinworm. Using the pinworm-specific ELISA we established, sentinel mice as well as randomly chosen mice from stocks and their off-spring are routinely screened for anti-*S. obvelata* antibody. Mice which test positive in the ELISA are removed from their cage, killed and their intestine examined for worms. If confirmed positive by the presence of worms, all mice within the cage are killed and discarded. It is highly likely that if one mouse is infected, all mice maintained in the same cage are infected. The spread of infection is kept to a minimum by using individually-ventilated cages (IVCs) providing a sterile environment within the cage which is independent from neighboring cages and the surrounding room.

If the mice are important such as breeding stock, and discarding them is not an option, the mice may be quarantined and treated for pinworm. It would be advised to use only F2 generation mice after treatment because IgG antibodies do pass from mother to child. Although it is dependent on the severity of the parents' pinworm infection there is a possibility that the F1 generation might still have circulating anti-*S. obvelata* IgG antibody and that these might interfere with an experiment.

Mice that are house in other facilities or imported should be housed in quarantine for 3 months. They may be tested for adventitious pathogens such as pinworm but should be treated regardless before being moved into an SPF animal facility

3.6 Reference

1. Jacoby RO, Lindsey JR: Risks of Infection among Laboratory Rats and Mice at Major Biomedical Research Institutions. Ilar J 1998, 39:266-271
2. Chan KF: Life cycle studies on the nematode Syphacia obvelata. Am J Hyg 1952, 56:14-21
3. Lewis JW, D'Silva J: The life-cycle of Syphacia muris Yamaguti (Nematoda: Oxyuroidea) in the laboratory rat. J Helminthol 1986, 60:39-46
4. Stahl W: Studies on the life cycle of Syphacia muris, the rat pinworm. Keio J Med 1963, 12:55-60
5. Rats ColDoMa, Resources IoLA, Sciences CoL, Council NR: Digestive System Infectious Disease of Mice and Rats, The National Academic Press, 1991, pp 85-163
6. Abimiku AG, Dolby JM: The mechanism of protection of infant mice from intestinal colonisation with Campylobacter jejuni. J Med Microbiol 1987, 23:339-344
7. Barthold SW, Beck DS, Smith AL: Mouse hepatitis virus and host determinants of vertical transmission and maternally-derived passive immunity in mice. Arch Virol 1988, 100:171-183
8. Arango-Jaramillo S, Wisseman CL, Jr., Azad AF: Newborn rats in the murine typhus enzootic infection cycle: studies on transplacental infection and passively

acquired maternal antirickettsial antibodies. Am J Trop Med Hyg 1988, 39:391-397

9. Rodrigues MdC, Torres JA, Zlotnik H: Investigation of humoral immune response in a murine model of actinomycetoma. Puerto Rico Health Science Journal 1996, 15:91-95

10. Bachmann MF, Odermatt B, Hengartner H, Zinkernagel RM: Induction of long-lived germinal centers associated with persisting antigen after viral infection. J Exp Med 1996, 183:2259-2269

11. Prescott L, Harley JP, Klein DA: Medical Microbiology. USA, Wm. C. Brown Publishers, 1996

12. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W: Current Protocols in Immunology. USA, John Wiley & Sons, Inc., 2006

13. Bier J, Ohanian SH: [Bleomycin-induced tumor cell lysis caused by antibodies and complement]. Dtsch Zahnarztl Z 1980, 35:102-104

14. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (editors): Current Protocols in Immunology. USA, John Wiley & Sons, Inc., 2006

15. Hall WC, Ganaway JR, Rao GN, Peters RL, Allen AM, Luczak JW, Sandberg EM, Quigley BH: Histopathologic observations in weanling B6C3F1 mice and F344/N rats and their adult parental strains. Toxicol Pathol 1992, 20:146-154

16. Weisbroth SH, Peters R, Riley LK, Shek W: Microbiological Assessment of Laboratory Rats and Mice. Ilar J 1998, 39:272-290

17. http://www.biosciences.drexel.edu/Homepage/immunology:

18. Sinha N, Mohan S, Lipschultz CA, Smith-Gill SJ: Differences in electrostatic properties at antibody-antigen binding sites: implications for specificity and cross-reactivity. Biophys J 2002, 83:2946-2968

19. Breiteneder H, Mills C: Structural Bioformatics Approaches to Understanding Cross-Reactivity. International Bioinformatics Workshop. Spain, 2005

20. Breiteneder H, NMills C: Structural Bioformatics Approaches to Understanding Cross Reactivity, Spain, 2005 International Bioinformatics Workshop

21. Lozano MJ, Martin HL, Diaz SV, Manas AI, Valero LA, Campos BM: Cross-reactivity between antigens of Anisakis simplex s.l. and other ascarid nematodes. Parasite 2004, 11:219-223

22. Robinson M, Gustad TR, Erickson MR: Immunological interactions between Trichinella spiralis and Heligmosomoides polygyrus: cross reactivity between muscle larvae and antibodies raised to unrelated antigens. Int J Parasitol 1997, 27:865-872

23. Fan CK, Su KE: Cross-reactions with Ascaris suum antigens of sera from mice infected with A. suum, Toxocara canis, and Angiostrongylus cantonensis. Parasitol Int 2004, 53:263-271

24. Jenkins SN, Behnke JM: Impairment of primary expulsion of Trichuris muris in mice concurrently infected with Nematospiroides dubius. Parasitology 1977, 75:71-78

25. Alghali ST, Hagan P, Robinson M: Hymenolepis citelli (Cestoda) and Nematospiroides dubius (Nematoda): interspecific interaction in mice. Exp Parasitol 1985, 60:364-370

26. Behnke JM, Wakelin D, Wilson MM: Trichinella spiralis: delayed rejection in mice concurrently infected with Nematospiroides dubius. Exp Parasitol 1978, 46:121-130

27. Sato Y, Ooi HK, Nonaka N, Oku Y, Kamiya M: Antibody production in Syphacia obvelata infected mice. J Parasitol 1995, 81:559-562

28. Kohler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975, 256:495-497

29. Barile MF, Hopps HE, Grabowski MW: Mycoplasma Infection of Cell Cultures. New York, Plenum Press, 1978, pp 35-45

30. McGarrity GJ: Advances in Cell Culture. Edited by Maramorosch K. New York, Academic Press, 1982, pp 99-131

31. McGarrity GJ, Kotani H: The Mycoplasmas. Edited by Razin S, Barile MF. New York, Academic Press, 1985, pp 353-390

32. McGarrity GJ, Vanaman V, Sarama J: Cytogenetic effects of mycoplasmal infection of cell cultures: a review. In Vitro 1984, 20:1-18

33. Gautschi EK, Baumann M, Eggel A, Marti P, Muller L, Truffer R, Schaub A, Schegg V, Wynmann S: Antibodies: Isolation, Labeling and ELISA. Presented at the Immunology II Practical Course, Institute of immunology, university of Bern, Switzerland, 2005-2006

34. Keeling JE: Experimental trichuriasis. I. Antagonism between Trichuris muris and Aspiculuris tetraptera in the albino mouse. J Parasitol 1961, 47:641-646

Conclusion

In this study, we showed for the first time the Th-type 2 immune response induced by *S. obvelata*, the mouse GI nematode. Resistance was predominately IL-13-dependent with a minor role for IL-4 in the expulsion of *S. obvelata* worms. We reiterate what others have shown that *S. obvelata* is a potential threat to experimental research influencing most animals kept within conventional facilities. The pervasive parasite induced a non-protective immune response to ovalbumin-induced anaphylaxis and produce antigens that are both cytotoxic and inhibitory to host cells. Nevertheless, early detection for rapid elimination is possible by an ELISA, which is both sensitive and inexpensive.

Future research

An interesting finding was the complete resistance observed in naturally infected 129/Sv mouse strains. Mice experimental infected with 500eggs and analyzed 35 days later similarly controlled the infection. Contrasting to the resistant BALB/c and C57BL/6 mouse strains, no worms were recovered from the cecum of 129/Sv mice. Two possibilities would need to be investigated. Firstly, in a kinetic experiment we have to determine whether this strain rapidly expel the GI nematode, and/or secondly whether the mice are completely resistant to reinfection which we believe accounts for the low worm burden observed later during infection in BALB/c and C57BL/6 mice.

S. obvelata infection of cell-specific IL-4R$\alpha^{-/-}$ mice demonstrated that IL-4 and IL-13 signaling on macrophages, T cells (only IL-4 signaling, no response to IL-13 in mice) and smooth muscle cells are not required for pinworm expulsion. Identifying the dominate cells involved will help unravel the mechanism(s) employed by the host to defend itself from GI nematodes. We have recently acquired epithelial cell specific IL-4R$\alpha^{-/-}$ mice, which we will analyze in our *S. obvelata* infectious model. Epithelial cells have been shown to play an important role early in the expulsion of *T. muris*.

To further optimize the specificity, sensitivity, and develop a reproducibly sustainable ELISA we will aim to isolate, purify and concentrate the four molecules (65, 64, 60, 58kDa) identified in our Western blot against *S. obvelata* antigen. From these, monoclonal antibodies will be generated for use as diagnostic tools and further characterize the molecules.

Syphacia obvelata antigen was shown to be both inhibitory and cytotoxic. However, further investigation is required to elucidate the modulatory effect which could explain the influences observed in mice concomitantly infected with pinworm.

Acid alcohol

1% HCl in 70% ethanol

30% acrylamide / 0.8% bisacrylamide

30g/100ml Acrylamide
0.8g/100ml Bisacrylamide
100ml dH_2O
Mix and filter solution through a 0.45μm filter
Store at 4°C up to 1 month

*** acrylamide is a neural toxin work under fumehood ***

Anaesthetic

1.2ml	Anaket-V (100mg/ml) (Centaur labs, Isando)
0.8ml	Rompun (2%) (Bayer, Germany)
8.0ml	PBS (1×)

Filter sterile and store in dark at 4°C

Blocking Buffer

4% BSA	40g	→ Alternatively: 2%	20g Milk Powder
0.02% NaN_3	0.2g		
1× PBS	100ml of 10× PBS		

Make up to 1L and store at 4°C

Blocking serum

150μl rabbit serum in 10ml 1× PBS

Coating Buffer

0.02% NaN_3 0.2g

1× PBS

Coating Buffer for mMCP-1 (carbonate buffer)

8.401g/L NaHCO3

dH_2O

pH 9.6

Make up to 1L and store at RT

Carnoy's fluid

60% Ethanol (100%)

30% Chloroform

10% Glacial acetic acid

Store at room temperature

Concanavalin A

Reconstitute stock of 5mg into 5ml sterile PBS (1mg/ml)

Store as 100μl aliquots at -20°C

Dilution Buffer

1% BSA 10g

0.02%NaN_3 0.2g

1× PBS

Make up to 1L and store at 4°C

Dilution buffer for mMCP-1

20ml PBS (10×)
8g BSA
dH_2O

Make up to 200ml Store at 4°C

Discombes stain

10ml Acetone
10ml 1% Eosin
80ml dH_2O
3ml 0.1M EDTA

Store at Room temperature

DMEM

1 tube powdered medium
10% FCS
3.7g $NaHCO_3$
5ml Penicillin/Streptomycin 200×

Then make up to 1L
Filter sterilise and store at 4°C

DMEM-20

20%	FCS
3.7g	$NaHCO_3$
110mg	Na pyruvate
1×	Penicillin/Streptomycin (stock:200×)
10mM	HEPES
1×	Non-essential amino acids (stock: 100×)

Then make up to 1L

Filter sterilise and store at 4°C

DMEM-20-HAT

1:50 HAT (reconstituted stock in 10ml media)

Filter sterilise and store at 4°C

Ischove's media (IMDM)

1 tube powdered medium

81.7ml	$NaHCO_3$ (10×)
5ml	Penicillin/Streptomycin (200×)

pH 7.2-7.3

Make up to 1L with dH_20, filter sterile and store at 4°C

Supplement with 200mM L –Glutamine before use

L-Glutamine

Working stock : 200mM

= 2.922g / 100ml ddH_2O

Use 1% in medium

Store at –18°C

6× loading buffer

25%	BMP blue
0.25%	Xylene Cyanol
50%	Glucose

Store at 4°C

LPS

Reconstitute stock of 1mg into 1ml sterile PBS (1mg/ml)

Store as 100μl aliquots at -20°C

o-Phenylenediamine (OPD)

3g/L OPD

330μl H_2O_2

1L Tris Triton

Make up fresh

P.A.S stain (Schiff's reagent)

Dissolve 1g paraosaniline in 200ml dH_2O (boiling) in a stopped flask;
Shake for 5min and allow to cool (50°C) filter, then add 20ml HCl;
Allow to cool further (25°C) and add 1g sodium or potassium metabisulphite;

Store O/N (18-24hrs) in the dark followed by the addition of 2g activated charcoal and 1min shaking. Filter – Store in dark (0-4 °C)

10× PBS (stock)

		Final Conc. 1× PBS
80g NaCl	1.37M	(0.137M)
2g KCl	0.027M	(2.7mM)
14.4g Na_2HPO_4	0.095M	(9,5mM)
2.4g KH_2PO_4	0.018M	(1.8mM)

Adjust pH to 7.4 and filter.

For tissue culture – autoclave
For anything else – filter sterile

For use dilute stock 1 in 10 (1× PBS)
Red cell lysis buffer (RCLB)

Dissolve,
8.34g NH_4Cl
0.037g EDTA
1g $NaHCO_3$
1L ddH_2O

Filter sterilise
Store at 4°C

PEG/DMSO

Prepare fresh, melt and mix;

PEG 4000 solution with DMEM-0 (50% w/v) at 56°C

Filter mixture through a 0.22μm filter and add Dimethylsulfoxyde (DMSO) at a 6:1 ratio (PEG 50%:DMSO)

Protease inhibitor cocktail

2.0mM AEBSF
1.0mM EDTA
130mM Bestatin
14mM E-64
1.0mM Leupeptin
0.3mM Aprotinin

Use diluted 1/25

RPMI medium 1640

1 tube powdered medium
900ml/L dH_2O
$NaHCO_3$ 2g/L
Penicillin/Streptomycin (100×) 10ml/L

pH 7.35
Filter sterilise

Running buffer (5×) for PAGE gels

Dissolve,
15.1g TRIS

72g	Glycine
5g	SDS
1L	ddH_2O

Store at RT

Alkaline phosphate buffer (Substrate buffer)

(Dilute your substrate in this buffer)

0.2g	NaN_3
97ml	di-ethanolamine (liquefy in water bath)
0.8g	$MgCl_2.6H_20$

Add 700ml H_2O

Adjust the pH to 9.8 with HCl

Then make up to 1L and store at 4^oC

Substrate for mMCP-1

Mix equal volumes of:

Peroxidase Solution A and;

Peroxidase Solution B

Transfer buffer

Tris	3.02g/L
Glycine	14.4g/L
Methanol	20%

Store at 4^oC before use

Tris –acetate buffer (50×)

242g	Tris
57.1	glacial acetic acid
100ml	0.5M EDTA

Make up to 1L with dd H_2O

Tris –borate buffer (5×)

54g	Tris
27.5ml	boric acid
20ml	0.5M EDTA

4× Tris·Cl/SDS, pH 6.8

Dissolve,

Tris base	6.05g/100ml
DH_2O	100ml

pH 6.8

Filter solution through a 0.45μm filter before adding

SDS	0.4g/100ml

Store at 4°C up to 1 month

4× Tris·Cl/SDS, pH 8.8

Dissolve,

182g/L	Tris base
1L	dH_2O

pH 8.8

Filter solution through a 0.45μm filter before adding

4g/L SDS

Store at 4°C up to 1 month

Make up to 1L with dd H_2O

Trypan blue dye

0.4% Trypan blue solution

0.81% sodium chloride

0.06% potassium phosphate

Washing Buffer (20×)

20g	KCL
20g	KH_2PO_2
144g	$Na_2HPO_4.2H_20$
800g	NaCl
50ml	Tween 20
100ml	10%NaN_3

Make up to 5L with dd H_2O

Washing Buffer

20ml PBS (10×)

0.5ml Tween-20

1× PBS

Make up to 1L and store at RT

Western blot staining solution

1 NBT/BCIP tablets

10ml dH_2O

Vortex to completely dissolve.

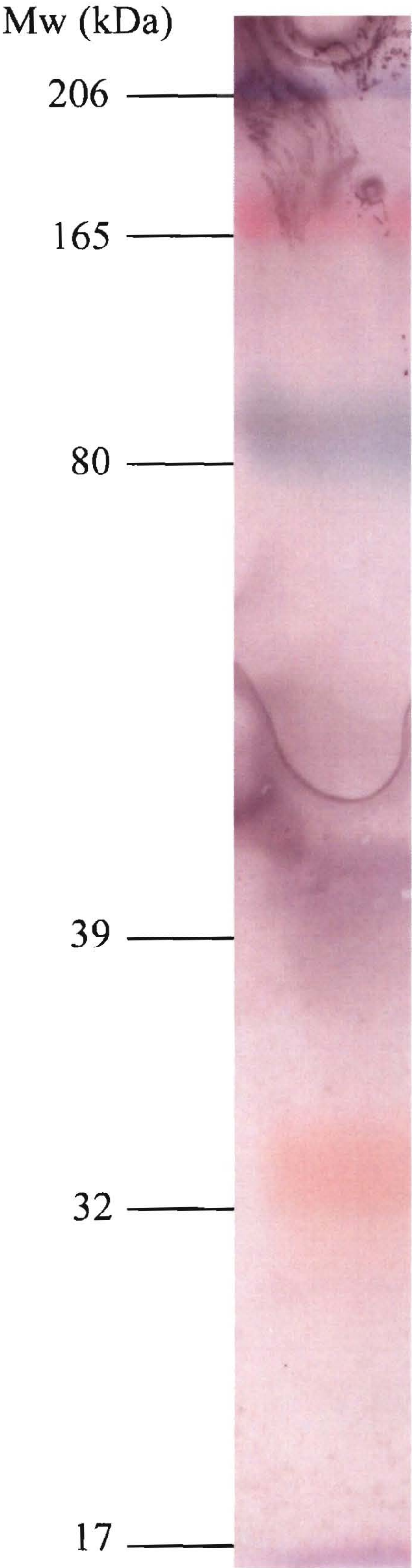
Store tablet at 2-8°C

Secondary Antibody for western blot

150μl normal mouse serum

50μl biotinylated IgG

10ml 1× PBS


Mw (kDa)
206
165
80
39
32
17

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