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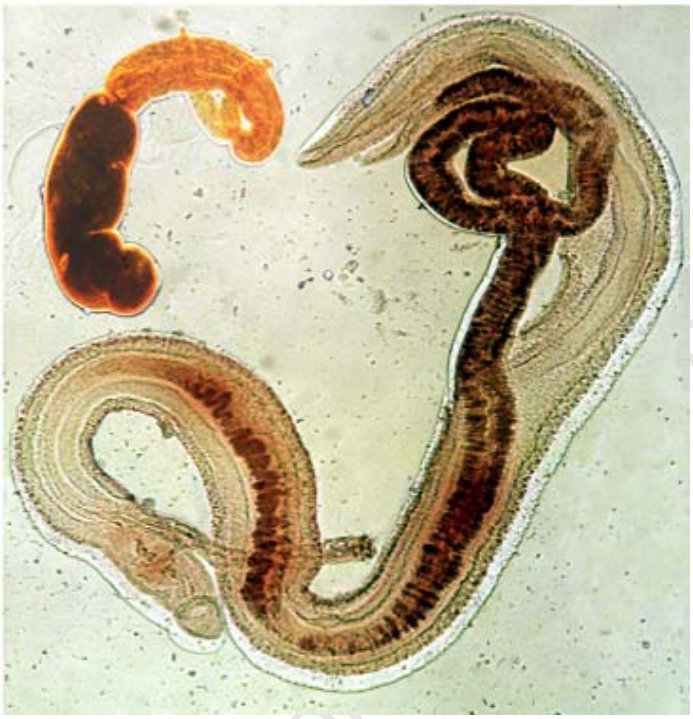

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Declaration

I, Reece Gerrad Marillier, hereby declare that the work on which this thesis is based, is my original work (except where acknowledgements indicate otherwise) and that neither the whole or any part thereof is being, has been or is to be submitted for another degree in this or any other University.

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Reece Gerrad Marillier

February 2008

Dedication

This thesis is dedicated to THE ONE who does all things well!

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List of Symbols and Abbreviations

Ab – antibody

Ag – antigen

AMMΦ- Alternatively activated macrophages

AP – alkaline phosphatase

APC – antigen presenting cell

APES – 3-aminopropyltriethoxysilane

Arg-1 – arginase-1

BSA – bovine serum albumin

CAB- Chromotrope 2R and aniline blue

CDC – Centre for Disease Control

CO_2 – carbon dioxide

Cre – Cre recombinase

DC – dendritic cell

ddH_2O – double distilled water

DEPC – diethyl pyrocarbonate

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

FACS – fluorescence-activated cell sorting

Fig. – Figure

Foxp3- Forkhead box p3

g – gram(s)

GM-CSF- Granulocyte colony stimulating factor

HRP – horseradish peroxidase

H&E – haemotoxylin and eosin

H_2O – water

Ig – immunoglobulin

IFNγ – interferon gamma

IL – interleukin

IL-13Rα1 – interleukin 13 receptor alpha 1

IL-13Rα2 – interleukin 13 receptor alpha 2

IL-4Rα – interleukin-4 receptor alpha

INOS – inducible nitric oxide synthase

i.p. – intraperitoneal

IRS – insulin receptor substrate

i.v. – intravenous

IVC – individually ventilated cages

JAK – Janus kinase

KO – knockout

L – litre

Lck – lymphocyte specific tyrosine kinase

LPS- Lipopolysacchararide

LysM – Lysozyme M

M – molar

MHC – major histocompatibility complex

mg – milligram

mg/ml – milligrams per millilitre

ml – millilitre

MLN- meseteric lymph node

mM – millimolar

MMCP-1 – mouse mast cell protease-1

mol – moles

mRNA – messanger RNA

n – number

N – nolar

ng – nanogram

ng/ml – nanograms per millilitre

NK – natural killer

nm – nanometre

NO – nitric oxide

NOS-2 – nitric oxide synthase 2

OCT – oxacalcitriol

OD – optical density

P – probability

PAS – periodic acid-Schiff

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PNP – 4-nitrophenyl phosphate

PRR – pattern-recognition receptor

RANTES – Regulated on Activation, Normal T-cell Expressed and Secreted

RNA – ribonucleic acid

rpm – revolutions per minute

RT-PCR – reverse transcriptase polymerase chain reaction

SA – somatic antigen

SCF – stem cell factor

SD- standard deviation

SEA- soluble egg antigen

Sec – seconds

SEM – standard error of the mean

sp – species

SPF – specific pathogen free

spp. – species (plural)

STAT – signal transducer and activator of transcription

TCR – T cell receptor

TGF-β – transforming growth factor beta

T_H – T helper cell

T_H1 –T helper cell type 1

T_H2 – T helper cell type 2

Treg – T regulatory cell

UCT – University of Cape Town

U.K. – United Kingdom

U.S.A. – United States of America

WT – wildtype

WHO – World Health Organization

Wk – week

x – times

α – alpha

β – beta

γ – gamma

γc – common gamma chain

γg – microgram

µg/ml – micrograms per millilitre

µl – microlitre

^{0}C – degrees celsius

% – percent

< – less than

-/- – knockout

Publications

1. IL-4/IL-13 independent goblet cell hyperplasia in gastrointestinal helminth infection
Marillier R, Michaels C, Smith E, Fick L, Dewals B, Leeto M, Horsnell W Brombacher F, BMC immunology 2008 March 9:11

2. TH1-dominant granulomatous pathology does not inhibit fibrosis or cause lethality during murine schistosomiasis.
Leeto M, Herbert DR, **Marillier R**, Schwegmann A, Fick L, Brombacher F. Am J Pathol. 2006 Nov;169(5):1701-12.

Manuscript in Preparation:

1. Smooth muscle cells IL-4/IL-13 responsiveness is necessary for successful passage of *schistosoma mansoni* eggs during murine infection. **Marillier R**, Horsnell W, Dewals B, Leeto M, Cutler T, Brombacher F

2. IL-4 responsiveness inhibits peripheral CD4+CD25+FoxP31 + regulatory T cell induction but induces IL-10-secreting and CD4+CD103+FoxP3− 2 T cells during helminth-driven inflammatory responses. Dewals B, Hoving J, **Marillier R**, Leeto M, Cutler A, Horsnell W, Brombacher F.

ABSTRACT

Schistosoma mansoni egg passage through intestinal tissue into the faecal stream is a critical event for completion of the life cycle of the helminth and involves induction of a type 2 immune response, which is necessary for the host's survival. Where as T cell-specific IL-4Rα has been shown not to be essential for survival, macrophage/neutrophil-specific IL-4 receptor α-deficient mice ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$), mice lacking alternative macrophages (AAMΦ), had severe intestine pathology and high endotoxin levels due to poor egg excretion and dysregulated granuloma formation. This resulted in increased mortality.

To determine the role of AAMΦ in granuloma we aimed to describe *S.mansoni* egg induced granuloma formation in the presence and absence of AAMΦ using $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice. In vitro IL-4Rα is required for expression of eosinophil chemotactic factor (YM-1) and mannose receptor (MMR). In contrast in vivo and during live infection $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice peritoneal cells and granuloma express YM-1 and MMR-1 independently of macrophage specific IL-4Rα deletion. However the localisation of YM-1 and MMR positive cells around the eggs, was dependant on the presence of AAMΦ. In the absence of macrophage specific IL-4Rα and therefore AAMΦ, classically activated macrophages (iNOS positive) accumulated closely around the eggs and there was an associated increase in T cells in the granuloma of $IL\text{-}4R\alpha^{-/-}$ and $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to WT mice. We suggest that IL-4Rα on macrophages is necessary for the localisation YM-1/MMR positive cells in the granuloma of *S.mansoni* infected mice which mediates immune suppression.

S.mansoni is known to induce hypercontractility of the intestine during infection. The aim was to characterise smooth muscle IL-4Rα deficient mice ($SM\text{-}MHC^{cre}IL\text{-}4R\alpha^{-/lox}$) during acute *S.mansoni* infection in order to determine if the IL-4/IL-13 responsiveness is required on SMC's during infection. $SM\text{-}MHC^{cre}IL\text{-}4R\alpha^{-/lox}$ mice had an intact TH2 immune response during infection. However

SMC-specific IL-4Rα was required for egg expulsion. These mice were more susceptible to infection, with transiently increased morbidity and mortality that was not due to sepsis. Further investigation is required to determine if the increased egg burden is due to decreased contractility and if this results in exacerbates port hypertension which may lead to mortality.

Similarly to grastointestinal motility, induction of goblet cell hyperplasia is associated with T_H2 immune responses. Here we examine the goblet cell hyperplasic response to three experimental parasitic helminth infections; namely *Nippostrongylus brasiliensis*, *Syphacia obvelata* and *Schistosoma mansoni*. The data demonstrated *S.mansoni* can induce intestinal goblet cell hyperplasia can be independent of T_H2 immune responses associated with parasitic helminth infections.

S.mansoni granuloma formation and expulsion of eggs is a coordinated event that requires expression of IL-4Rα on both smooth muscle cells and macrophages in order to reduce the burden created by continual production of eggs by *S.mansoni* worms in the mesenteric veins of the intestine.

Literature Review .

LR.1 Introduction

Life expectance has risen over the last century in the developed world, this has been driven largely by the design of vaccines and drugs that are effective against many of the known bacterial, viral and helminth infections. The progress that has been made, however, is hampered by growing poverty especially in the developing world (Editorial, 2007). Poor drug delivery and education in health care systems lead to increased drug resistance, morbidity and mortality. In addition the growing burden of HIV and AIDS has left many diseases, namely tropical diseases, neglected. While the World Health Organisation and others are making an effort to drive attention towards these diseases and the plight of people affected, the scientific community is vigorously investigating the interaction of these diseases with the host immune system, to improve drug and vaccine development (http://www.who.int/neglected_diseases/en/, 2008).

The immune system has developed to recognize substances that belong to the host and foreign material which does not belong to the host (Janeway, 1992; Roitt *et al.*, 2001). The immune system then mediates the removal of the foreign material from the host. Among foreign substances, pathogens like bacteria, viruses or parasites are the main targets of the immune system. The immune response reacts against pathogens in two subsequent phases: (i) innate and (ii) adaptive immune responses (Rautava & Walker, 2007; Roitt *et al.*, 2001). Depending on the type of material, one or both of these arms of immunity are triggered. These responses involve cell-cell interaction and the production of mediators namely cytokines, chemokines, antibodies and enzymes.

The innate immune response is based on non-specific mechanisms of defense recognizing a wide range of pathogens and forming the first line of defense after the host physical barriers (Iriti & Faoro, 2007; Roitt *et al.*, 2001). It involves

humoral and cellular mediators. The humoral mediator of innate immune response is mainly the complement system (Markiewski & Lambris, 2007). The importance of this system is seen no more clearly in individuals with a genetic deficiency that leads to abrogation of the complement system (Sjoholm *et al.*, 2006). The complement system attacks the foreign invaders directly and damages the membrane of the pathogen. Myeloid cells represent the major cellular type involved in innate cellular immunity. Macrophages, neutrophils and natural killer cells may deal with the pathogen by phagocytosis, degranulation of cytotoxic mediators or killing of infected cell, respectively.

Cells of immune system recognize the host's environment by cell surface receptors, namely pattern recognition receptors (PRR)(Akira *et al.*, 2006). PRRs including Toll-like receptors and C-Type lectins, recognize and bind pathogen associated molecular patterns (PAMPS) (Ansel *et al.*, 2006; Iwasaki & Medzhitov, 2004; Janeway, 1992). These receptors bind and mediate the uptake and transport of PAMPS to endosomes where they are processed before antigen presentation by antigen presenting cells (APC). APCs are dendritic cells, monocytes, macrophages, neutrophils, and B-cells (Ploegh, 2007; Roitt *et al.*, 2001).

APCs provide a connection between the innate and the adaptive immune response (Creusot & Mitchison, 2004; Steinman, 2006). Dendritic cells (DC's) are the chief APC. A review by Perona-Wright et al. (2006) describes that there are three specific immune activated states for DC. These activated states lead to polarization of the immune response: (i) conventionally mature DC's which respond to intracellular pathogens (bacteria, viruses and protozoa) leading to Type 1 immune response; (ii) tolerogenic DC's which respond to apoptotic cells and commensal bacteria (leading to a tolerogenic phenoType) and (iii) modulated DC (mDC) that respond to helminth infection. The mDC's represent a discrete activated class of cells that express a distinct set of co-stimulatory and signalling molecules and generate a Type 2 immune response. DC migrate from the area

of antigen exposure to the draining lymph node where they present antigen to naïve T cells (Guermonprez *et al.*, 2002; Ploegh, 2007).

The adaptive response as the name suggests improves upon repeated exposure to the same antigen, and is therefore said to have memory (Boskovic *et al.*, 2006), (Kapsenberg, 2003). Memory is medaiated by cell cellular and humoral responses which are driven by lymphocytes include T and B cells, respectively (Mitchison, 2004). B cells produce antigen-specific antibodies upon activation through antigen and T helper cells. T cells are activated by APC through T-cell receptor (TCR) triggering. Upon activation, T cells differentiate into T helper (T_H) or cytotoxic T cells (CTL). CTL are involved in controlling viral infections and other intracellular pathogens and express the CD8 determinant. T_H cells expressing CD4 can differentiate into two main pathways, T_H1 and T_H2 (Mosmann *et al.*, 1986). T_H1 cells are involved in immune responses against intracellular pathogens. T_H2 cells develop mainly in response to extracellular parasites. Our understanding of polarisation however is no longer restricted to the T_H1/T_H2 dichotomy. T cells can also be stimulated to differentiate into regulatory T cells (T_{reg})that are involved in immune suppression and T_H17 cells which have been linked to defense against extra cellular bacteria, auto immune diseases and cancer (Tato *et al.*, 2006) (Figure A).

T_H cell polarization is driven by cytokines and T_H cells can be characterised by their response to cytokines, gene expression and the cytokines they produce (Tato & O'Shea, 2006). T_H1 cells are regulated by the transcription factor T-bet and signal transducer of transcription-4 (STAT-4) to produce the cytokines interferon gamma (IFN-γ) and interleukin (IL)-12 (Mosmann & Coffman, 1989; Park *et al.*, 2005). T_H2 cells are regulated by the transcription factors STAT-6, GATA-3 and c-maf to produce the cytokines IL-4, IL-13 and IL-5. T_{reg} cells are regulated by the transcription factor Foxp3 and are stimulated by TGFβ. T_H17 cells are activated by IL-6, TGFβ, IL-23 and are regulated by the transcription

factor STAT 3 to produce IL-17 and also IL-10 in certain inflammatory conditions (Jankovic & Trinchieri, 2007; Romagnani, 2006; Tato & O'Shea, 2006).

T_H2/Type 2 polarisation by cytokine/antibody production can be either harmful during allergy or beneficial as in helminth infection. *Schistosoma mansoni* is a strong inducer of Type 2 immune response and is for this reason an excellent model for understanding the T_H2 immune response (Pearce, 2004).

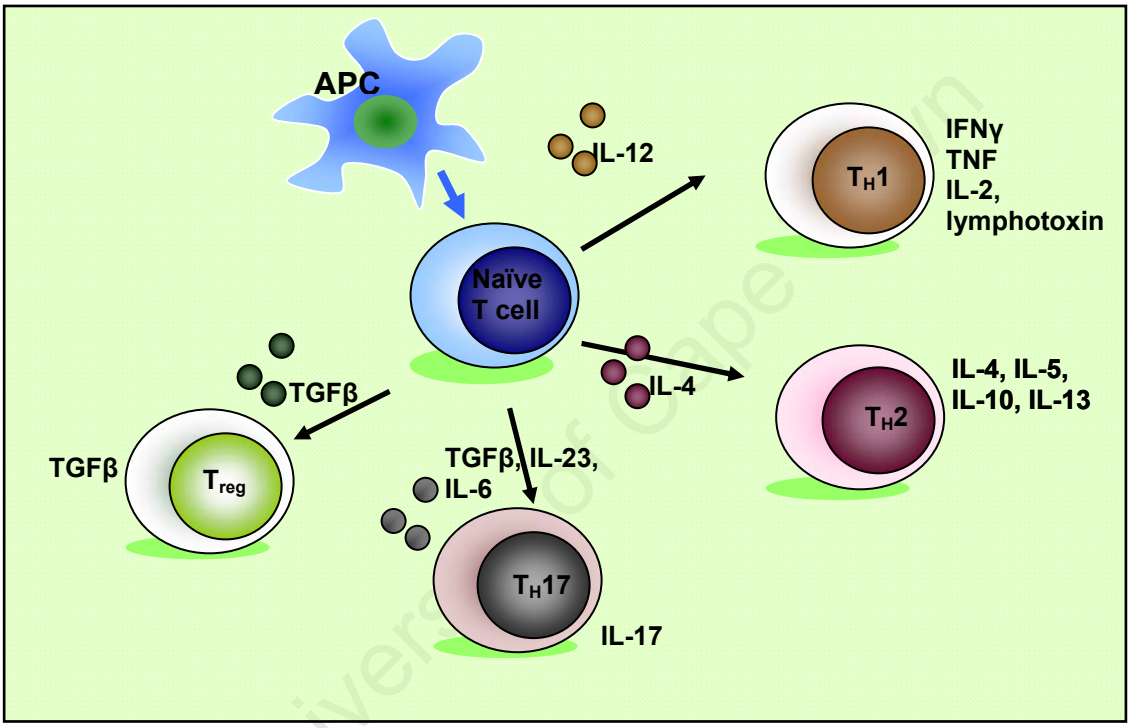


Figure A : Schematic representation of the differentiation of murine TH cells into subsets with distinctive patterns of cytokine release (adapted from (Dong, 2006))

LR. 2. TH2 cytokines

LR.2.1. Interleukin- 4

IL-4 is a pleiotropic cytokine first discovered by Maureen Howard and William Paul (Dubois *et al.*, 1998; Howard & Paul, 1982). IL-4 was initially described as a co-stimulator of B cells (BSF-1) (Dubois *et al.*, 1998; Howard & Paul, 1982). The source of IL-4 for initial polarisation of the Th2 response is still not clear. One possibility is that naïve T cells are signaled to express IL-4 upon binding to Jagged ligand on DC's via T cell notch receptor (Noben-Trauth *et al.*, 2000). A second possibility is that during some parasitic infections macrophages and mast cell produce IL-25/IL-17E which stimulates another cell, possibly an innate cell, to produce IL-4 (Coffman & von der Weid, 1997). After initial activation a number of cells produce IL-4 including specialized T cells (expressing NK1.1 and γδ T cells), $CD4^+$ T cells, basophils, mast cells and eosinophils and are thought to help with the polarisation process (Ansel *et al.*, 2006).

IL-4 Receptor

The effects of IL-4 are mediated via the IL-4R. By using radiolabeled mouse recombinant IL-4, Lowelthal et al. (1988) found that IL-4 binds specifically and with high affinity to a receptor on haematopoietic and non-haematopoietic cells in vitro (Lowenthal *et al.*, 1988). This receptor is present on monocytes, macrophages, T cells, neutrophils, endothelial, muscle, fibroblast, hepatocytes and brain tissue (Chiaramonte *et al.*, 2003). It has been found to be constitutively expressed by eosinophils (Dubois *et al.*, 1998).

IL-4 may bind to both Type 1 and Type 2 IL-4 receptor complexes. Type 1 IL-4 receptor contains two subunits: (i) the 140 kDa IL-4Rα chain and (ii) a 60 kDa γ-chain (the γ-chain of the IL-2 receptor) (Russell *et al.*, 1993). The Type 2 IL-4 receptor is a heterodimer composed of IL-4Rα and IL-13Rα1 (also known as IL-13R). Upon IL-4 binding the units heterodimerise and activate intracellular

signaling pathway involving the JAK kinases (Andrews *et al.*, 2002; Takeda *et al.*, 1997). Stat6 is phosphorylated by Jak, which is translocated to the nucleus for activation of transcription of IL-4 inducible genes (Andrews *et al.*, 2002; Singh & Agrewala, 2006) .

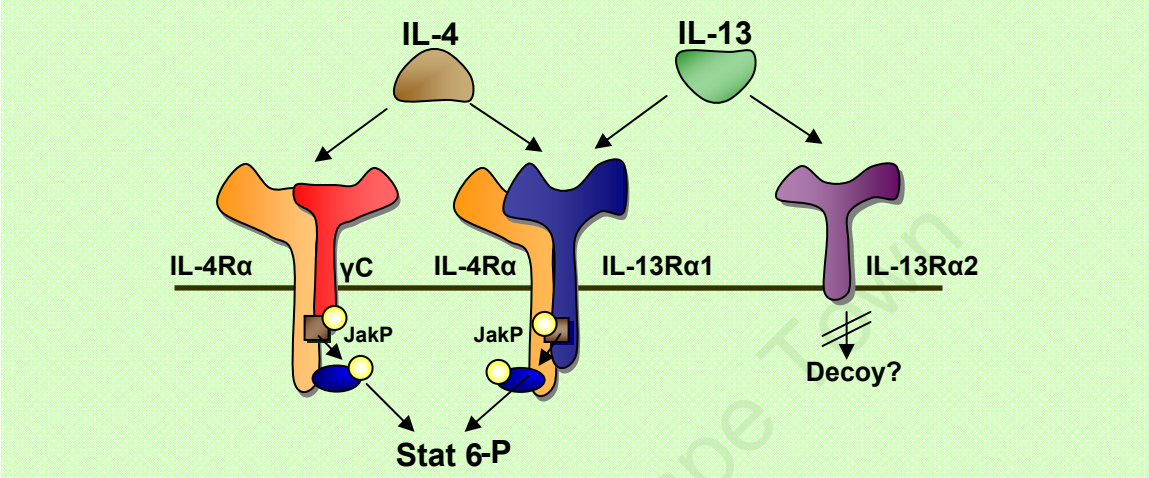


Figure B: IL-4/IL-13 signaling pathways

The Role of IL-4.

IL-4 regulates the humoral and the adaptive response by increasing the production of IL-4, IL-5, IL-9, IL-10 and IL-13 (Beckmann *et al.*, 1992; Mosmann & Coffman, 1989). As mentioned above IL-4 has an important role in B cell activation. IL-4 is responsible for growth of B cells, prolongs B cells lifetime and increases the expression of MHC-II and CD23 on their surface (involved in antigen presentation) (Keegan *et al.*, 1989). IL-4 is also essential for B cells immunoglobulin class switching and leads to production of the Type 2 antibodies IgE and IgG1 (Barner *et al.*, 1998; Coffman *et al.*, 1993) .

TH2 cytokines are important for the suppression of pro-inflammatory TH1 cytokines IFN-γ, IL-1, IL-6 and TNF and therefore act to decrease inflammation (Ansel *et al.*, 2006). However intradermal injection of IL-4 also causes rapid accumulation of eosinophils and therefore IL-4 is necessary for the chemotactic

response (Dubois *et al.*, 1998). IL-4 has also been implicated in leukocyte migration to inflammatory sites by induction of adhesion molecules (VCAM) on epithelial cells (Fukushi *et al.*, 2000; Thornhill *et al.*, 1991). IL-4's role in mast cell growth and activation has also been recognised (Bischoff *et al.*, 1999). Mice administered anti-IL-4 and anti-IL-13 antibodies failed to induce mastocytosis upon infection with a helminth (Madden *et al.*, 1991). IL-4 and IL-13 were also implicated in mast cell degranulation in a STAT 6 dependant manner (Madden *et al.*, 2002). The histamine and prostaglandin E2 (PGE2) released during mastocytosis have an effect on intestinal epithelium secretory functions (increasing ion and fluid flow in the gut lumen) resulting in helminth expulsion (Else & Finkelman, 1998). IL-4 and IL-13 also affect the activation state of macrophages.

LR.2.2 Interleukin 13

IL-13 shares many of the functions of IL-4 such as activation of IgE synthesis and the inhibition of pro-inflammatory cytokines. This is due to sharing of the IL-4Rα chain in their receptor complexes. However mouse T cells do not have a functional IL-13 receptor and thus are not involved in induction of T_H2 cell differentiation (Brombacher, 2000; Minty *et al.*, 1993)

The IL-13 receptor is expressed on human B cells, basophils, eosinophils, mast cells, endothelial, fibroblasts, monocytes, macrophages, respiratory epithelial cells and smooth muscle cells (Wynn, 2003). There are two IL-13 receptors, IL-13Rα1 which forms a complex with IL-4Rα (as mention above) and IL-13Rα2. Type 2 IL-4R (IL-13R) on non-haemopoietic cells is the main mechanism by which they respond to IL-4/IL-13. IL-13Rα2 is soluble and has mostly been thought to have suppressive decoy functions, however recent studies indicate that it my mediate Stat6 independent events such as fibrosis and TGFβ production (Chiaramonte *et al.*, 2003; Fichtner-Feigl *et al.*, 2006).

The biological roles of the TH2 responce have been considered a “double-edged sword” (Brombacher, 2000), they are involved in pathology and morbidity in some diseases such as allergy but promote resistance in helminth diseases such as schistosomiasis in acute phases and pathology in chronic stages of disease. What is most interesting, is that the immunological events of allergy also occur during helminth infection however they confer morbidity in the former and protection in the later. In allergy, antigen presentation to T-cells drives their release of IL-4, IL-13, IL-5 and IL-9, which stimulates B cell class switching to IgE, mastocytosis and eosinophilia when they bind to their receptors (Corry & Kheradmand, 1999). Inturn cross linking IgE on the surface of mast cells results in degranulation and release histamine. Histamine and other factors released by these cells result in events such as goblet cell hyperplasia, airway/ gastrointestinal hyperresponsiveness and fibrosis, which result in the symptoms of allergy. Futhermore invents such as fibrosis result in permanent changes to tissue function. In support of this role for the Th2 immune response, administration of bacterial components which drive TH1 responses such as lipopolysaccaride (LPS), Bacille Calmette-Guerin (BCG) and CpG-DNA have been shown to decrease allergic responses (Wohlleben & Erb, 2001).

IL-13 and IL-4 have many overlapping functions. Many of the roles for IL-13 and IL-4 have been demonstrated by the use of gene deficient mice, namely IL-4 and IL-13-/- as well as Stat $6^{-/-}$ (Barner *et al.*, 1998; Beckmann *et al.*, 1992; Schroder *et al.*, 2002) Our laboratory has developed a number of cell specific IL-4Rα gene deficient mice. Using the Cre recombinase/loxP system we have generated macrophage/neutrophil-specific IL-4Rα knock out ($LysM^{cre}IL\text{-}4R\alpha^{-/flox}$), smooth muscle cell-specific IL-4Rα knock out ($SM\text{-}MHC^{cre}IL\text{-}4R\alpha^{-/flox}$) and T cell IL-4Rα knock out ($Lck^{cre}IL\text{-}4R\alpha^{-/lox}$) mice (Herbert *et al.*, 2004; Horsnell *et al.*, 2007; Leeto *et al.*, 2006). The characterization of these mice in different inflammatory settings allows the determination of the essentiality of IL-4/IL-13 during inflammation. These mice show impaired TH2 responses for example impaired airway hyperreactivity and mucus production in allergy and fail to resolve

nematode infections and show increased susceptabiltity to schistosomiasis (Herbert *et al.*, 2004).Until recently, it has been difficult to separate the roles of Type1 IL-4Rα and Type2 IL-4Rα. However, the development of IL-13Rα1$^{-/-}$ mice has illustrated that the Type2 IL-4Rα receptor and hence IL-13 has a unique role during allergy and nematode infection (Ramalingam *et al.*, 2008). This is supported by earlier studies where overexpression of IL-13 resulted in anaphylaxes i.e. rapid and severe onset the of allergic response, independent of IL-4 (Fallon *et al.*, 2001). Ramalingam *et al*. (2008), studies also demonstrated an important role for Type1 IL-4Rα and hense IL-4 in the events of other helminth infections such as *Schistosoma mansoni* infection. In this review we focus on the role of T_H2 cytokines, IL-4 and IL-13, their origin and function and mechanisms in *S.mansoni* infection.

LR.3 *Schistosoma mansoni*

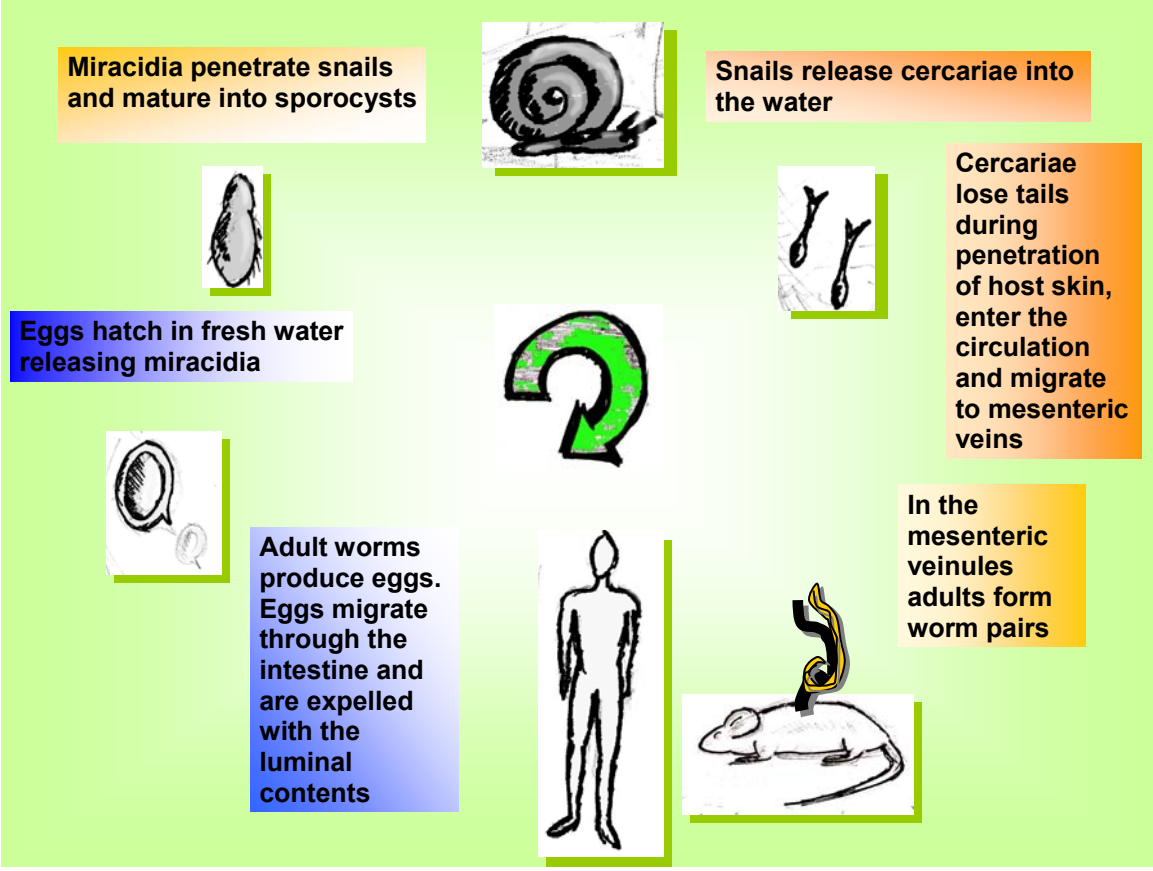
LR.3.1 Epidemiology

Schistosoma is a trematode blood fluke that causes the disease schistosomiasis. According to WHO more than 200 million people are infected and more than 700 million are at risk, most of whom live in the developing world (Chitsulo *et al.*, 2000). Theodire Bilharz first identified the helminth in Egypt in 1851. Since then schistosomiasis (Bilharzia) has been decribed as a major human tropical disease (Pearce & MacDonald, 2002). Five species infecting humans have been identified: *S. japonicum, S. haematobium, S.intercalatum, S.mekongi, S. mansoni.* For this review the focus is on *S. mansoni.*

Life cycle of *Schistosoma mansoni*

The life cycle of *S. mansoni* involves an intermediate host (an aquatic snail, *Biomphalaria glabrata*) and a definitive host (human). Humans become infected with *S.mansoni* when they come into contact with infected water. Cercariae from

infected water enter the host through the pores in the skin. The cercariae shed their tales during this process. Schistosomula enter the host circulation and eventually reach the mesenteric vessels were they differentiate and mature into egg producing adult worms (Armstrong, 1965). The female may dissociate from the large male and migrate further down the veins so that eggs are released in closer proximity to the intestine. One female can release about 300 eggs per day (Fitzsimmons *et al.*, 2004).



FigureC. Adult worm pair and Life cycle: (a) Female worm lying in the gynecophoral grove of the male. (b). Sketch of the life cycle of *S.mansoni* (adapted from the CDC website)

Eggs pass through the intestinal wall to reach the lumen where they are excreted with the hosts' faeces. Miracidia emerge from eggs in fresh water and infect the snail host. Inside the snail they mature differentiate and proliferate. Approximately four weeks post infection cercariae are released into the water and the life cycle continues.

LR.3.2. Pathogenesis in the mammalian host

S.mansoni is also known as gastrointestinal schistosomiasis. While the adult worms reside in the mesenteric veinules most of the symptoms are driven by egg deposition in the tissues. Eggs expulsion is not always successful, about 50% of the eggs become trapped in the intestine (Weinstock, 1992). Due to blood flow a proportion of the eggs are also shunted to the liver and sometimes to the lungs where they also become trapped (Rocha *et al.*, 1995; Weinstock & Boros, 1981). In the tissue trapped eggs induce inflammation which is characterised by granuloma formation (Weinstock, 1992). These large granuloma (approximately 1mm in diameter) cause a dramatic change to the intestinal morphology: the villi become broad and flat, goblet cell hyperplasia increases the amount of mucus secreted and each egg is accompanied by localized thickening of the muscle layer due to smooth muscle cell hyperplasia (Bogers *et al.*, 2000; Fallon *et al.*, 2000b). These changes are associated with symptoms namely diarrhoea, abdominal pain and intestinal bleeding (Guyatt *et al.*, 1995; Moreels *et al.*, 2001). Granuloma fibrosis causes enlargement and hardening of the liver and the spleen (hepatosplenic schistosomiasis) (Vennervald *et al.*, 2004). Fibrosis also occurs in and around portal veins and lead to portal hypertension during *S. mansoni* infection. However portal hypertension may also occur independently of fibrosis, particularly in children, which can be fatal (Njenga *et al.*, 1998). The mechanism by which these eggs pass through the mucosa remains unknown, however since immune compromised humans and mice fail to clear eggs, egg transit is considered an immune dependent process (Doenhoff, 1997).

LR.3.3 Immunobiology of *Schistosoma mansoni*

Much of understanding of the host's immune response has been gained from animal models. Here we describe the information gained from murine models of schistosomiasis. Initially the immune response to *S.mansoni* worms is a weak Type 1 immune response (zero to 4 weeks post infection) (Pearce *et al.*, 1991). Eggs deposited in the tissue however, drive the production of essential Th2 cytokine production, which peaks at week 8 and decreases from 12 weeks post infection into the chronic phase of infection (Grzych *et al.*, 1991; Pearce *et al.*, 2004).

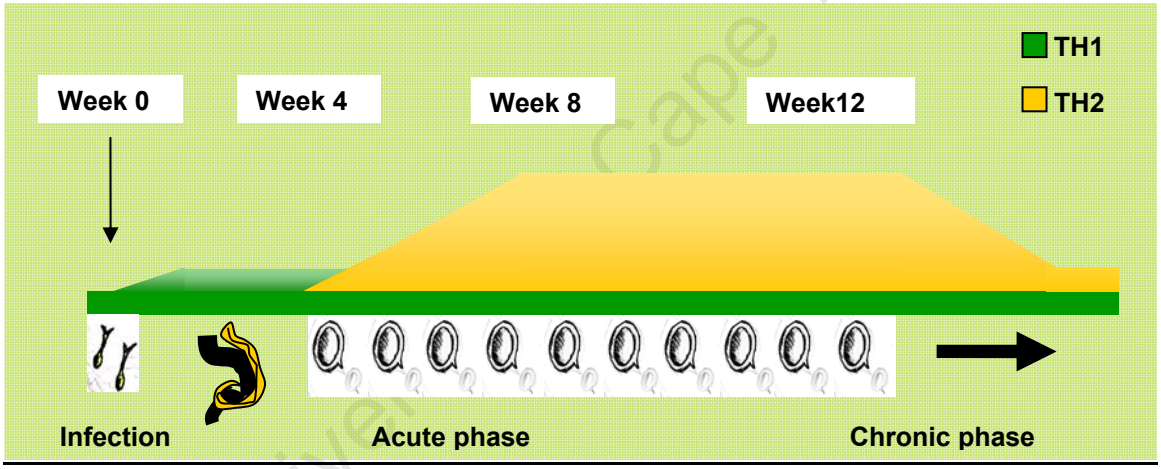


Figure D: Immunobiology of *S.mansoni* infection. Infected mice develop weak Th1 response. When eggs are produced by adult a strong Th2 response it marks the beginning of the acute phase which peaks at week 8. Chronic phase develops from approx. 12 weeks post infection.

Granuloma formation is considered to be the result of TH2 immune response. Granuloma are induced in most helminth infections (Pearce & MacDonald, 2002). In support of this, STAT6, IL-4/IL-13 or IL-4Rα deficient mice have poor granuloma formation, abrogated egg expulsion and severe gut immunopathology

which lead to sepsis (Fallon *et al.*, 2000a; Herbert *et al.*, 2004). These mice are unable to drive efficient TH2 response and succumb to infection during the acute phase.

When granuloma do not form in immune compromised individuals or in $CD4^+$ T cell deficient mice or as mentioned above TH2 deficient mice, egg expulsion is abrogated (Davies *et al.*, 2001; Doenhoff *et al.*, 1981; Dunne *et al.*, 1983; Pearce & MacDonald, 2002). This leads to the postulation that granulomas may mediate egg expulsion perhaps by recruiting motile cells that move the egg out into the lumen (Damian, 1987). About 2/3 of the eggs remain trapped in non-immune-compromised host tissue and therefore a more appropriate role for granuloma formation could be to destroy the eggs and prevent harmful substances from the egg damaging the host tissue. However granuloma also cause damage to the organs due to IL-13 induced fibrosis. Down modulation of granuloma formation is important in survival of the host during the chronic phase (Mentink-Kane *et al.*, 2004). Since the TH2 Type immune response induces granuloma formation and is considered to be anti-inflammatory/regulatory, granuloma formation may be protective in the short term by allowing egg expulsion and dampening inflammation but harmful when the infection becomes chronic and/or the egg burden becomes high (Pearce & MacDonald, 2002).

Eggs release a number of soluble factors, soluble egg antigen (SEA) which is highly glycosylated and easily recognized by C-Type lectins (CLRs). Human and mouse studies have shown that antigens (glycans, lipids and dsRNA) bind to TLRs and CLR's (for example mannose receptor) on the surface of DC's (van Liempt *et al.*, 2007a). Van Liempt et al. (2007) showed that co-binding of SEA to mannose receptor and TRL ligand was necessary to drive activation of monocyte derived DC's towards TH2(van Liempt *et al.*, 2007a). This response is necessary to directs the immune response towards granuloma formation (Cass *et al.*, 2007; Perona-Wright *et al.*, 2006).

Cells of the granuloma: The response to eggs results in the recruitment of many cells to the granuloma that vary according to the region (liver or intestine) and duration of infection (Weinstock & Boros, 1981). Early granuloma formation is charcterised by the recruitment of eosinophils and fibroblasts. Mature granuloma however contain large macrophages that accumulate close to the egg, few eosinophils, neutrophils, T and B lymphocytes, some fibroblast and mast cells (Bogers *et al.*, 2000). Mature granuloma of the liver also have a high level of fibrosis but this was not described in the intestine (Cheever *et al.*, 1995; Njenga *et al.*, 1998).

The exact role however for granuloma cells has not been defined. Although **eosinophils** are present in early granuloma formation and highly responsive to IL-4, they do not appear to have an essential role. Following infection of eosinophil deficient mice (ΔblGATA and TgPhil) no effect on granuloma formation, morbidity or mortality in these mice was observed (Swartz *et al.*, 2006).

T helper cells: In absence of $CD4^+$ T cells, such as in AIDS patients, *S. mansoni* infection causes a fatal outcome during the acute phase (Doenhoff *et al.*, 1981; Dunne *et al.*, 1983) This increased susceptibility has been associated with a poor TH2 development. However Leeto et al. (2006) found using T cell-specific IL-4Rα deficient mice that granuloma where able to form and these mice were less susceptible to *S. mansoni* infection. Therefore although $CD4^+$T cells are required, the development of TH2 response may not be essential for granuloma formation and susceptibility to *S. mansoni* infection.

Macrophages: "Elicited Macrophages" as opposed to resident macrophages in the tissue may have different activation states depending the type of immune response (Gordon, 2003) IL-4/IL-13 responsive macrophages were defined as alternatively activated (AAMΦ). AAMΦ have a distinct function(s) from classically activated macrophages, which respond to IFN-γ (review by (Gordon, 2003). The

activation of Mϕ by these Th2 cytokines drive the expression of AAMϕ markers like resistin-like alpha (Fizz-1), eosinophil chemotactic factor ECF-L (YM-1), arginase and mannose receptor (Gordon, 2003; Linehan *et al.*, 2003; Stahl, 1963).These markers provide a valuable tool to identify these AAMϕ. Linehan et al. (2003) showed that IL-4Rα signaling is essential for mannose receptor expression by granuloma macrophages during schistosomiasis (Linehan *et al.*, 2003). Herbert et al. (2004) further showed that mice unable to develop AAMΦ fail to excrete eggs efficiently and succumb to infection before WT mice (Herbert *et al.*, 2004).

While it has been shown that macrophages accumulate close to the eggs in granuloma the exact function of these AAMϕ has not been defined (Bogers *et al.*, 2000; Damian, 1987; Herbert *et al.*, 2004).

Non-haematopoietic cells. While the necessity of IL-4/IL-13 responsiveness on haematopoietic cells has clearly been demonstrated, direct evidence for a role of IL-4/IL-13 responsiveness on non-haematopoietic cells has not been described during schistosomiasis. The role of mucosal immunity during infection is gaining increasing attention. *S. mansoni* infection causes a number of changes to the host tissue during infection, many of these processes such as fibrosis and mucous production all involve non-haematopoetic cells. Three plausible candidates for involvement in schistosomiasis include epithelial cells, which may mediate migration of eggs and infiltration of inflammatory cells, fibroblasts, which make a more likely source of collagen and smooth muscle cells for egg expulsion (Madden *et al.*, 2002; Moreels *et al.*, 2001; Murata *et al.*, 1999).

Smooth muscle cells (SMC). *S.mansoni* has been shown to induce increased proliferation of smooth muscle cells and increased response of these cells to contractile agents, namely hypercontractility. Moreels showed that stat-6 deficient smooth muscle cells from *S.mansoni* infected mice have reduced response to contractile agent (Moreels *et al.*, 2001). Since Stat-6 is the downstream signalling

molecule of IL-4Rα, the results suggest that IL-4/IL-13 responsiveness is required for increased contractility during schistosomiasis. However the effect of this mutation on *S.mansoni* egg transit and associated inflammation has not been examined.

The nematode *Nippostrongylus brasiliensis* infection generates a Th2 cytokine response from the host for effective expulsion of the parasite. Recently Horsnell *et al.* (2007) showed that during *Nippostrongylus brasiliensis* infection, mice lacking IL-4Rα on smooth muscle cells (SM-MHC^{cre}IL-4Rα$^{-/lox}$) have delayed goblet cell hyperplasia and worm expulsion as well as reduced expression of muscarinic receptor-3 (M3). Since M3 plays a role in driving smooth muscle contractions it was suggested that reduced expression of the receptor results in decreased contractility and consequently in delayed worm expulsion. From these results it could be proposed that IL-4/13 responsive smooth muscle cells may be essential for driving contractility, goblet cell hyperplasia and perhaps egg expulsion during schistosomiasis (Bogers *et al.*, 2000; Horsnell *et al.*, 2007; Moreels *et al.*, 2001).

It is clear from the literature that both haematopoetic cells are required for egg expulsion and granuloma formation. However the exact roles for their IL-4/IL-13 responsiveness are not known. In this study we attempt to directly determine the role of IL-4/IL-13 responsive macrophages, smooth muscle cells and specialized epithelial cells (goblet cells) during the acute phase schistosomiasis.

OBJECTIVES OF THE PROJECT

- To determine the role of IL-4/IL-13 responsive macrophages in granuloma formation during *Schistosoma mansoni* infection.
- To determine the role of IL-4/IL-13 responsive smooth muscle cells during *Schistosoma mansoni* infection.
- To determine the role of IL-4/IL-13 during goblet cell hyperplasia induced by helminths, *Nippostrongylus brasiliensis*, *Syphacia obverlata* (pin worm) and *Schistosoma mansoni*

MATERIALS AND METHODS

MATERIALS AND METHODS

MM.1 ANIMAL WORK

Mice were kept in the Health Science Faculty animal unit of the University of Cape Town (UCT), in individually ventilated cages under specific-pathogen-free (SPF) conditions. All experiments were performed in accordance with guidelines laid down by the Animal Ethics Research Board of UCT (Cape Town, South Africa). All mice were aged between 6 to 10 weeks and sex matched for each experiment.

MM.1.1 Generation and genotyping of conditional IL-4Rα-deficient mice

IL-$4^{-/-}$ (Noben-Trauth *et al.*, 1996)IL-4/$13^{-/-}$(McKenzie *et al.*, 1999)and IL-4R$\alpha^{-/-}$ (Mohrs *et al.*, 1999) SM-MHC^{cre}IL-4R$\alpha^{-/lox}$ (Horsnell *et al.*, 2007) LysM^{cre}IL-4R$\alpha^{-/lox}$ (Herbert *et al.*, 2004) mice were generated on a BALB/c background. Transgenic negative littermates (IL-4Rα-/lox) also referred to as wildtype (WT) were used as controls in all experiments. Genotypes of the mice were confirmed routinely in our lab by PCR analysis of tail biopsies under the following conditions : 94^{o}C/1 minute, 94^{o}C/30 seconds, 57^{o}C/30 seconds, and 72^{o}C/1 minute for 40 cycles on an MJ thermocycle (Biozym Diagnostik, http://www.biozym.com, Hessissch Oldenhof, Germany). Specific PCR primer pairs are shown in Table 1 below:

TABLE 1. Primer pairs for confirmation of mice genotypes

Gene	Primers
IL-4Rα	5'-GTACAGCGCACATTGTTTTT-3'
Deletion	5'-GGCTHCCCTGGAATAACC-3'
	5'-CCTTTGAGAACTGCGGGC-3'
LoxP	5'-CCCTTCCTGGCCCTGAATTT-3'
	5'-GTTTCCTCCTACCGCTGATT-3'
Cre	5'-ATGCCCAAGAAGAAGAGGAAGGT-3'
	5'-GAAATCAGRGCGTTCGAACGCTAGA-3'

MM. 2 PARASITES AND INFECTION

MM. 2.1 *Schistosoma mansoni*

Schistosoma mansoni (S.mansoni) of the Puerto Rican strain, infected and non-infected snails (Biomphalaria glabrata) were originally obtained from Adrian Mountford (York, UK). The life cycle of *S.mansoni* is maintained in our laboratory at 25^{o}C under regulated 12 hour Light and 12 hour dark cycles in Lepple water and fed on Tetra Pond food sticks (Melle, Germany http:www.tetra.net).

MM.2.1.1 Live Infections

Mice were infected as previously described in Current Protocols in Immunology (Coligan *et al.*, 1991). Naïve sex-matched mice from 6 to 10 weeks of age were anaesthetised with Anaket-V (Centaur Labs, RSA) and 2% Rompun (Bayer, Germany) at recommended concentrations and the abdomen clean shaved. The abdomen was the washed with conditioned water. Animals were placed on their backs in a slotted restraining device to prevent involuntary movement from disturbing the infection process; Cercariae were shed from snails and their numbers adjusted to the required concentration (Coligan *et al.*, 1991). Stainless steel rings were placed on their abdomens in the shaved area of the mice and the cercarial suspensions pipetted into the rings with a Pasteur pipette. Animals were exposed to cercariae for 30min and the cercarial suspension and the rings were removed. Mice were then moved back into their cages without washing or wiping the exposure area.

MM 2.1.2 Detemination of schistosome eggs burden from mouse tissues

Ileum and colon were removed 2 cm proximal and 0.5 cm distal to the caecum, respectively (McDermott *et al.*, 2005).The tissue was weighed and digested in 5ml of 5% potassium hydroxide overnight at 37 $^{\circ}$C. The digests were vortexed and centrifuged at 100g for 5min to pellet eggs. The supernatant was aspirated

until 1-2mls remained. The eggs were vortexed and counted in 50 µl in triplicate. The counts were presented as eggs per gram of tissue as previously described (Keiser *et al.*, 2006; Kloetzel, 1967).

MM 2.1.3 Determination of schistosome eggs expulsion from mouse faecal material

Analyses of feacal egg samples for the presence of eggs was performed using the modified Kato-katz technique as described by (Doenhoff & Bain, 1978; Eberl *et al.*, 2002). Briefly: 10-40mg faecal pellets were suspended in isotonic solution overnight. The pellets were disrupted by aspiration in a syringe without a needle and filtered through a 150µm mesh sieve. Each sample was counted in triplicate using an inverted light microscope at (100x magnification), values were averaged and normalised according to the re-suspension volume and the weight of the faeces collected.

MM 2.2 *Syphacia obvelata*

Syphacia obvelata infections were maintained and performed by Dr. C. Michel (UCT, South Africa): The following methods were provided:

Infection and recovery of *S. obvelata* were performed as previously described (Stahl, 1963). Briefly, eggs of *S. obvelata* used for infection were collected from the caeca of naturally infected mice (IL-4/13$^{-/-}$, and IL-4Rα$^{-/-}$) maintained in barrier facilities. The caeca were collected in 0.65% NaCl, cut open, and submerged in a gauze mesh at the mouth of a conical flask for 1 to 2 h at 37°C to allow the worms to migrate out. Worm burdens were assessed on various days post infection. After being washed in 0.65% NaCl, worms were crushed and their eggs were isolated by passage through 70µm nylon cell strainers (BD Falcon, BD Biosciences, Belgium). Each mouse was inoculated orally with 500 eggs using oral dosing cannulae (VetTech, Cheshire, United Kingdom).

MM 2.3 Nippostrongylus brasiliensis

Nippostrongylus brasiliensis infection was maintained and performed by Dr. W. Horsnell. The following methods were provided. *N. brasiliensis* nematodes were kindly provided by Klaus Erb, (Wurzberg, Germany). Mice were subcutaneously injected with 750 L3 larvae of *N. brasiliensis*. Analysis of numbers of adult worm numbers in the intestine was determined as previously described (Horsnell *et al.*, 2007).

M.3 ENZYME LINKED IMMUNOSORBENT ASSAY

M.3.1 Cytokine and Antibody analysis

Analysis of antigen-specific IgG1, IgG2a and b, IgE and cytokine determination production was carried out by capture–enzyme-linked immunosorbent assay (ELISA). In brief, Nunc Maxisorp 96 well plates (Nalge Nunc International, Naperville, IL, USA) were coated with 10ug/ml of "capturing antibody" or 10ug/ml of *S.mansoni* soluble egg antigen (SEA) overnight at 4^{o}C in borate buffer (50mM), pH 9.6. Plates were washed with washing buffer using a Skan Washer 300 microplate washer (Skatron Instruments, Norway) and blocked with 200µl of blocking buffer overnight. Wells were washed and recombinant mouse cytokine or isotype specific antibody standards were added in serial dilutions at recommended concentrations. Samples were diluted in dilution buffer and added in serial dilutions and the plates were incubated overnight at 4^{o}C. Plates were washed and incubated overnight at 4^{o}C or 2 hours at 37^{o}C with biotinylated antibody. Plates were then washed and incubated with streptavidin-alkaline phosphatise (BD Pharmingen) (1:1000 dilution) for 1 hour at 37^{o}C. The plates were subsequently washed, incubated with p-nitrophenyl phosphate (1mg/ml) (Boehringer Mannheim, Germany) and the enzyme reaction was read at 405nm using a Versamax micropklate reader (Molecular Devices, Sunnyvale, CA, USA).

Parasite-specific antibodies were detected using alkaline phosphatase conjugated goat anti-IgG1 (B020-NK20), goat anti-IgG2b (F659-UF89), and rat anti-IgE (23G3) (all from Southern Biotechnology Associates). Total IgE was determined with monoclonal antibodies 84.1C and alkaline phosphatase-conjugated rat anti-IgE (23G3) for detection. For cytokines used see Table 2.

MM 3.2 Splenocyte and Mesenteric lymph node restimulation for cytokine analysis

Single cell splenocyte and mesenteric suspensions were prepared from spleens removed from infected at experiment end points and uninfected mice (Coligan *et al.*, 1991). $1x10^6$ splenocytes or mesenteric lymph node cells per ml were cultured in IMDM (Gibco) media supplemented with 10% fetal calf serum (Gibco) for 72h at 37^{o}C in 96 well plates pre-coated with either PBS or 20mg/ml anti-CD3 (clone 145-2C11) or SEA. Cells were then centrifuged at 1200rpm for 5min and the supernatants collected. Supernatant cytokine concentrations were then determined by ELISA as described previously (Mohrs *et al.*, 1999).

MM.4 Endotoxin assay.

Concentration of endotoxin in the serum was determined by using Limulus Amebocyte Lysate (LAL) QCL-1000® detection kit (Cambrex). Standard curves were performed with endotoxin-standard (Sigma). Briefly, sera were diluted 1:10 in endotoxin-free water and heated for 5 min at 75°C before endotoxin detection.

MM.5 FACS ANALYSIS

Bone marrow derived cells or Peritoneal Exudate Cells were stained for surface markers by flow cytometry. Stainings were performed in FACS buffer (0.1% BSA 0.02% NaN_3 PBS) containing 2.4G2 mAb (anti-FcγRII/III)

and 2% inactivated rat serum. Cells were stained during 15 minutes on ice with the following conjugated antibodies: CD11b-FITC (M1/70, BD), CD11c-biotinylated (BD), CD4-PE (GK1.5, BD), CD8-biotinylated (53.6.72), CD19-PE (1D3, BD), F4/80-PE (R&D), MMR-biotinylated (MR5D3, Serotec), goat anti-YM1-biotinylated (R&D). Unbound antibody was removed by centrifugation in 1ml FACS buffer. When applicable, pelleted cells were resuspended and incubated for a further 15 minutes on ice with 100μl of a saturating amount of streptavidin-APC (BD) in FACS buffer. Cells were further washed and resuspended in 500μl FACS buffer supplemented with 7-AAD (Sigma, St. Louis, USA). Stained cells were acquired using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, California, USA) and cells analyzed using CellQuest software (both BD). Dead cells stained with 7-AAD were excluded from analysis.

MM. 5.3 Isolation of Peritonal Exudate Cells (PEC) for FACs analysis

Mice were inoculated IP with *S.mansoni* eggs and sacrificed 7 days after infection. Peritoneal exudates cells harvested by flushing the peritoneal cavity with 10ml of sterile Iscoves's-Modified Dulbecco's Medium (IMDM) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (All Gibco, Invitrogen Corporation, Carlsbad, CA, USA). Harvested cells were centrifuged at 1200 rpm for 8 minutes and resuspended in 2ml complete IMDM supplemented with 10% FCS, 100 U/ml penicillin and 100μg/ml streptomycin (all Gibco). Cells in single cell suspension were counted using an improved neubauer haemocytometer. 5 x 10^5 cells were pooled for each experimental mouse group and were washed in FACS buffer (PBS supplemented with 0.1% BSA and 0.05% Sodium Azide) by centrifugation at 1200 rpm for 5 minutes. Using flow cytometry (*see section MM 5*) the expression of the AAMΦ markers (MMR and YM1) were analyzed on CD11b+F480+ gated population (Figure 2A).

MM.6 ISOLATION OF BONE MARROW DERIVED CELLS AND DIFFERENTIATION INTO MACROPHAGES

Bone marrow derived cells and differentiation into macrophages was performed at previously described (Schwegmann *et al.*, 2007). BMDMs were generated as described (Holscher *et al.*, 2006).

Method is quoted from Dr Anita Schwegmann (Thesis 2006 University of Cape Town): **"**Mice were sacrificed by cervical dislocation and the femur and tibia bones collected. The bone marrow cells were flushed from the bones using DMEM (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) containing 10% FCS, 100U/ml penicillin G and 100µg/ml streptomycin. The cells were washed and concentrated by centrifuging at 1200 rpm at 4^{o}C for 10 minutes. The bone marrow cells were added at a final concentration of 1 x 10^6 cells/ml in PLUTZNIK media (DMEM containing 10% FCS, 5% horse serum, 2mM L-glutamine, 1mM Na-pyruvate, 0.1mM 2-β-Mercaptoethanol, 30% L929 cell-conditioned medium, 100U/ml penicillin G, 100µg/ml streptomycin) and transferred into a special gas-permeable 15cm x 6cm Teflon coated-bag (Max Planck Institute for Immunobiology, Max Planck, Freiberg, Germany). The L929 conditioned medium contained GM-CSF and M-CSF, which stimulated the differentiation and growth of bone marrow stem cells into macrophages. The open end of the bag was heat-sealed and bone marrow stem cells incubated at 37^{o}C under 5% CO_2 for 10 days. The supernatant was discarded and the adherent cells were massaged from the Teflon-coated bags into DMEM containing 10% FCS, 100U/ml penicillin G, 100µg/ml streptomycin. The BMDMs were washed twice in DMEM containing 10% FCS, 100U/ml penicillin G, 100µg/ml streptomycin to get rid of residual GM-CSF from the L929 conditioned medium. The BMDMs were plated at a density of 5 x 10^5 BMDMs/ml and incubated at 37^{o}C under 5% CO_2."

L929 conditioned medium

L929 cells were maintained in DMEM (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) containing 10% FCS, 100U/ml penicillin G and 100µg/ml streptomycin until 90% confluence. The growth media was removed and the cells washed in 10ml 1 x PBS. Cells were removed from the plastic surface of the flask by incubation in 5ml Trypsin/EDTA (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) at room temperature for 4 minutes. The cells were washed in 50ml DMEM (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) containing 10% FCS, 100U/ml penicillin G, 100µg/ml streptomycin and seeded at 2 x 10^4cells/ml in 100ml DMEM (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) containing 10% FCS, 100U/ml penicillin G, 100µg/ml streptomycin. The L929 cells were grown in 162cm2 tissue culture grade flasks (Corning Costar Corporation, Cambridge, MA, USA) at 37^{o}C under 5% CO_2 for 7 days. The supernatant was harvested and centrifuged at 2500 rpm for 15 minutes at 4^{o}C to get rid of cell debris. The clear supernatants were stored in 50ml aliquots at –20^{o}C."

MM.7 MEASUREMENT OF NITRIC OXIDE IN CULTURE SUPERNATANTS

Cell culture supernatants were analyzed for the production of NO using the Griess reaction assay, which measures the concentration of nitrite, a stable product of the reaction of NO with O_2 (Lowenthal *et al.*, 1988). BMDM from IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were pre-incubated with medium or IL-4/IL-13 and subsequently stimulated with medium or IFNγ/LPS and supernatants were collected after 48hrs. Supernatant samples and standards (1mM NO_2 solution) were serially diluted three-fold in DMEM containing 10% FCS, 100U/ml penicillin G, 100µg/ml streptomycin (all Gibco, Invitrogen Corporation, Carlsbad, CA, USA) and 50µl added to designated wells in a flat bottomed 96 well plate. 25µl of Griess Reagent 1 (1% sulfanilamide in 2.5% phosphoric acid) and then 25µl of Griess Reagent (2 0.1% napthyl-ethylene-diamine in 2.5 % phosphoric acid) were sequentially added to each well. The plate was incubated at room

temperature for 5 minutes to allow the reaction to develop. The purple-pink colour of the reactions were read at A_{540nm} and the reference at A_{690nm} using VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, U.S.A).

MM.8 REAL-TIME RT-PCR

RNA was extracted from the intestine of infected mice 8 weeks PI, using Tri-reagent (Sigma), cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, http://www.promega.com). Samples were DNAse 1 treated to remove contaminating genomic DNA. Muscarinic receptor (M3) cDNA was amplified using the following primers: 5'-CGG AAA AGG ATG TCG-3' AND 5'-GGC ACT CGC TTG TGA A-3'. Nk1 cDNA was amplified using the following primers: 5'-CTG ACC AAT CGA GTC T-3' and 5'-CCA GTT ACC CGA ACC A-3'. Data were normalized using ribosomal protean S12 using the following primers 5'-GGA AGG CAT AGC TGC TGG AGG T-3' and 5'- CGA TGA CAT CCT TGG CCT GA-3' and β-actin using the following primers 5'- ACA TTC CCG CGG TGT AGA CA-3' and 5'-GAC AAA GAC CCA GAG GCC ATT-3' house keeping genes.

MM.9 IMMUNOPHATHOLOGY

MM. 9.1 Histology and histopathology

Tissue samples were fixed in a neutral buffered formalin solution. Following embedding in paraffin, samples were cut into 5-7μm sections. Sections were stained with haematoxylin and eosin (H&E), periodic acid Schiff reagent (PAS) or with chromotrope 2R and aniline blue solution (CAB) and counterstained with Wegert's haematoxylin for collagen staining. Photomicrographs were captured using a Nikon 5.0 Mega Pixels Color Digital Camera (Digital SIGHT DS-SMc). Colour balance and conversion of images to black and white where completed

using Adope Photoshop 7.0. Goblet cell number were assessed by counting the number of positively stained cells (PAS positive) per five villi or crypts was counted by light microscopy for small intestine or colon, respectively. All samples were randomized and counted in a blinded manner.

Granuloma were measured using "NIS elements BR Basic Research software, 4D experiment ability" (IMP Scientific & Precision (Pty) Ltd). The area (mm^2) of each granuloma containing a single egg was measured by subtracting the area of the egg from the area of the whole granuloma (mm^2) using the software indicated above (Cheever *et al.*, 1992). An average of 25 granulomas per mouse were measured and included in the analysis. All histological examinations were scored by the same individual in a blind fashion to obtain consistency.

MM.9.2 Immunohistochemistry

Formalin Fixed, Paraffin embedded tissue sections were cut into 7um sections. Tissue was rehydrate through Zylol. alcohol to water. Sections were blocked with 1% H_2O_2 in Methanol for 15 min and rinsed in distilled H_2O. Antigen retrieval was performed by heating sections in a Pressure Cooker for 2 min in 10mM Citrate Buffer pH6 and followed by a rinse PBST. Endogenous biotin was blocked using a the Avidin/Biotin blocking kit (Vector Laboratories, Southern Cross Biotechnology) : blocked with 0,1% Avidin for 15 min. Rinse PBST and blocked with 0,01% Biotin for 15 min. Sections were wash well in PBST and incubated with biotinylated antibody (see table of antibodies) for 90 min, wash PBST and incubate with HRP-Avidin 1:400 for 30 min. Unbound antibody was washed off with PBST. DAB Substrate was used for 10 min and washed well in tap water. Haematoxylin 4 min was used as a counterstain, Tap Water 5 min, Dehydrated through alcohols to Zylol and Coverslipped with Entellan (MERCK).

MM.9.3 Immunofluorescence

A Leica cryostat (Leica Intruments, Germany) was used to cut 7um sections from OCT (Tissue-Tek, Sakura Finetek, Europe) embedded frozen sections and mounted onto 3-aminopropyltriethoxysilane (APES) coated slides (B&D Germany), and dehydrated overnight at 4˚C (Conway & Kiernan, 1999). Slides were brought to room temperature and sections were fixed using 100% ice cold acetone for 10min. Slides were air dried for 5min and rehydrated by washing in the slide in NH_4Cl 50mM for 2min followed by 2 washes with Wash the slide in 0.05% Tween 20 (PBS) 3X 5min. 100ul of 1% BSA plus 10% NS (the serum that the secondary antibody is raised in) serum (PBS) was used to block non-specific binding for 30min by placing as much onto the slide as to cover the section (100-200ul). Primary antibodies were diluted in PBS (see table for list of antibodies) and place on the section using a pipette and incubated overnight at 4degree in a humidified chamber or 90min at room temperature. The antibody was flicked off and slides were washed 3X PBS 5 min. 100ul of diluted secondary antibody was pipetted onto each section and incubate at room temperature for 30min in the dark. Slides were washed in 3X PBS 5min and coverslipped using DAKO fluorescent mounting media containing antifade reagent (DakoCytomation, Dako North America Inc., USA).

Specific staining was determined by comparing positive stain to negative/ isotype controls. Both the liver and intestine are particularly difficult tissues to stain, the hepatocytes of the liver are auto-fluorescent and the intestine contains a number of cells that contain fluorescent granules and mucus which also binds a number of antibodies non-specifically. To overcome this fixed tissue was washed in ammonium chloride (50mM) to reduce auto-fluorescence, incubated with BSA to prevent binding due to charge, normal serum was used to block non-specific binding and endogenous biotin was blocked using a blocking kit (from Vector Laboratories©). Isotype control/non relevant IgG antibodies were then used to determine if the primary antibody was binding non-specifically to conserved epitopes. Once background staining was determined using photoshop all the

images were adjusted to the same levels to reduce fluorescent noise in the chosen images.

MM.10 STATISTICS.

Data are presented as means ± standard error of the mean (SEM), and the significant differences were determined using Student's *t* test (Prism software, http://www.prism-software.com).

MM. 11 ADDITIONAL REAGENTS

All chemical reagents used in these studies were of analytical grade and purchased from the following companies unless otherwise stated:
Sigma-aldrich, South Africa
BDH Chemicals Ltd. Poole, England
Merk Laboratory Supplies, South Africa

MM. 11.1 General reagents

MM. 11.1.1 10x Lepple water (for maintaining snails)

5.6g $CaCl_2$
12.28g $MgSO_4.7H_2O$
0.43g K_2SO_4
4.2g $NaHCO_3$
0.48mls of $FeCl_3.6H_2O$ solution (2.5g dissolve in 500mls H_2O)

Dissolve each of the ingredients in 1 liter of distilled water, join the ingredients except the $NaHCO_3$ and allow to stand for 1 hour add the $NaHCO_3$ and make up the solution to 10 liters. Working stock is then dissolved 1 part Lepple, 9 parts H_2O.

MM. 11.1.2 Phosphate Buffered Saline

8g NaCl

0.2g KCL

1.44g Na_2HPO_4

0.24g KH_2PO_4

Dissolve reagents in 900ml distilled H20. Adjust to pH 7.4 HCL. Adjust volume to 1000ml. Filter through a 0.22um filter (Millipore Corporation, Bedford, USA)

MM. 11.2 ELISA reagents

MM. 11.2.1 Antibodies

Table 2. Antibodies used in cytokine ELISA

Cytokine	Capturing antibody Clone number	Detection Antibody Clone number (biotinylated)	Source
IL-4	BVD4-1D11	BVD6-24G2	Pharmingen
IL-13	38213	Matched To capture Ab.	R&D
IL-10	JES5-2A5	SXC-1	Pharmingen
IFNγ	R4-6A2	XMG 1.2	Pharmingen
TGFβ	A75-2.1	A75-3.1	Pharmingen

MM. 11.2.2 Blocking buffer

40g Bovine Serum Albumin (Boehringer Mannheim, Germany)

0.2g NaN3

Dissolve BSA and NaN3 in 800ml PBS (pH 7.2), bring the final volume to 1000ml and store at 4oC.

MM. 11.2.3 Dilution Buffer

10g BSA

0.2g NaN3

Dissolve BSA and NaN3 in 800ml PBS (pH 7.2), bring the final volume to 1000ml and store at 4oC.

MM. 11.2.4 20x Washing Buffer

20g KCL

20g KH2PO4

144g NaH2PO4

800g NaCL

Dissolve reagents in 4500 distilled H2O. Add 50ml 10% NaN3. Bring final volume to 5000ml and store at room temperature.

MM. 11.2.5 Substrate Buffer

0.2g NaN3

0.8g MgCl2

Dissolve NaN3 and MgCl2 in 700ml distilled H20. Add liquefied diethanolamine and adjust the pH to 9.8. Bring the final volume to 1000ml with distalled H20. Sterilise and store in the dark at 4oC.

MM. 11.3 Immunohistochemistry reagents

Table 3: Histology antibodies

Cytokine	Capturing antibody Clone number	Source
B220 (CD45)	RA3 6B2	Pharmingen
CD3	145-2C11	
CD11b	M1/70	Pharmingen
CD204 FITC	MCA 1322F	Serotec
CD206 (MMR)	5D.3	Gift from Gordon Brown (South Africa)
YM1 (ECF-L)-biotinylated	NSO-derived rmECLF-L	R&D
iNOS	polyclonal	Abcam
MHCII-biotinylated	M5/114	

MM. 11.4 Tissue Culture Reagents

MM. 11.4.1 Iscove's Modified Dulbecco's Medium (IMDM)

Dissolve 1 tube (3.024g/L) of IMDM in 750ml distilled H2O. Add 81.7ml 10x NHCO3 and 2ml of 500x penicillin/streptomycin. Using 1N NaOH, adjust the pH to between 7.2-7.4. Bring the final volume to 1000ml with distilled H2O. Sterilize through a 0.22um filter (Millipore Corporation, Bedford, USA) and store at 4oC

MM. 11.4.2 Red Cell Lysis buffer

8.34g NH4Cl

0.037g EDTA

1g NaHCO3

Dissolve reagents in 1000ml distilled H20. Sterilize and store at 4^{o}C.

CHAPTER1

CHAPTER1

IL-4Rα expression on macrophages regulates their activation state and localization in *S.mansoni* induced granuloma

1.1 INTRODUCTION

In response to pathogen infection, two main types of macrophage activation states have been described (Gordon, 2003). Typically classically activated macrophages are activated during TH1 immune responses by IFN-γ, IL-12 and IL-18. These macrophages are involved in cellular immunity against intracellular pathogens for example *Mycobacterium* and *Listeria monocytogenes*. Classically activated macrophages produce compounds such as nitric oxide (NO) which has antimicrobial activities but have also been involved in tissue damage (Gordon, 2003).

Alternatively activated macrophages (AAMϕ) are activated during TH2 immune response by IL-4/IL-13 binding to the type I and II IL-4 receptor on the cell surface and therefore AAMϕ may be defined as IL-4/IL-13 responding macrophages (Gordon, 2003; Ramalingam *et al.*, 2008). AAMϕ are speculated to be involved in allergic, cellular and humoral responses to extracellular parasites and helminths (Gordon, 2003). From earlier studies it was not clear whether AAMϕ were responsible for protection or pathology, since they were often identified at regions of inflammation. Nevertheless they are now known to be suppressers of inflammation. With enhanced phagocytic ability AAMϕ take up apoptotic cells, reducing proinflammatory signals, they reduce T cell proliferation and produce factors necessary for wound healing (Goerdt & Orfanos, 1999). During *S. mansoni* infection, using macrophage/neutrophils specific-IL-4Rα$^{-/-}$ mice (LysM^{cre}IL-4Rα$^{-/lox}$), it has been shown showed that alternative activation of macrophages is necessary for survival during acute schistosomiasis (Herbert *et al.*, 2004). Although in absence of AAMϕ granuloma formation seems unaffected in the liver, infected mice succumb to infection during the acute phase of *S.*

mansoni infection. Dysregulated intestinal inflammation with endotoxaemia explained the high susceptibility of mice with macrophage specific impaired expression of IL-4Rα (Herbert *et al.*, 2004).

IL-4 responsive macrophages express a number of markers that have functional significance, namely arginase. In AAMϕ the production of arginase is stimulated by TH2 cytokines via the IL-4Rα. Arginase catalyses the conversion of L-arginine to L-ornithine and urea which results in the production of proline and the inhibition of NO production. While arginase has therefore been considered the chief AAMϕ marker (Gordon, 2003; Raes *et al.*, 2002), IL-4Rα signaling is essential for mannose receptor (MMR) expression by granuloma macrophages during schistosomiasis (Linehan *et al.*, 2003). MMR is a C-type lectin and together with Toll like receptors is involved with uptake of soluble *S.mansoni* egg antigen (SEA) by antigen presenting cells. SEA presentation to T-cell drives their differentiation into TH2 cells (van Liempt *et al.*, 2007b). IL-4 and IL-13 also drive the expression of additional markers including: the resistin-like α (Fizz-1) and chitinase-like molecules, eosinophil chemotactic factor ECF-L (YM-1) (Gordon, 2003; Linehan *et al.*, 2003; Stein *et al.*, 1992). YM1 is involved not only in chemotaxis, but also in-capsulate pathogens and interacts with the extracellular matrix for wound healing (Nair *et al.*, 2003). However the expression profile of AAMϕ markers may vary according to the inflammatory model. In addition the expression of these markers may be driven by other cytokines and factors such as IL-10 and glucocorticoids which confounds the identification of IL-4/IL-13 responsive macrophages which as stated above are AAMϕ/IL-4Rα expressing macrophages (Goerdt & Orfanos, 1999).

While it has been determined that AAMϕ have an essential protective function during acute schistosomiasis the exact role of these cells and mechanism/s by which they protect the host is not known. In this chapter we attempted to answer this question by examining the expression and localization of AAMϕ markers

within the acute phase *S.mansoni* granuloma in relation to the egg and other cell types in the presence and absence of IL-4Rα expression.

We showed that in vitro IL-4Rα$^{-/-}$ BMMϕ did not express YM1 and MMR, however in vivo SEA stimulated peritoneal cell from LysM^{cre}IL-4Rα$^{-/lox}$ mice and in granuloma from LysM^{cre}IL-4Rα$^{-/lox}$ mice expression of YM1 and MMR were not completely impaired. However by a macrophage-IL-4Rα dependant mechanism YM1^{+} and MMR^{+} cells accumulated close to egg. Failure to do this was associated with increased T cell proliferation in the gut and the liver granulomas.

1.2 RESULTS

1.2.1 IL-4 and IL-13 induce expression of YM1 and MMR on bone marrow-derived macrophages expressing IL-4Rα.

MMR and YM-1 expressed on the cell surface are markers of AAMϕ. AAMϕ marker/receptor expression may be mediated in vivo directly by pathogen associated factors or indirectly by the innate and adaptive immune systems. To determine whether IL-4 and IL-13 alone drive expression of MMR and YM-1, bone marrow-derived macrophages (BMMϕ) were generated from WT (IL-4Rα$^{-/lox}$) or IL-4Rα$^{-/-}$ mice and analyzed for expression of markers of AAMϕ.

Following differentiation, BMMϕ phenotype was determined by flow cytometry. BMMϕ were negative for all T-cell (CD4 and CD8), B-cell (CD19) or dendritic cell (CD11c) markers but expressed CD11b (Figure 1A). In order to further confirm their phenotype, macrophages from WT and IL-4Rα$^{-/-}$ were simultaneously stimulated with or without IL-4 and IL-13 and assessed for surface expression of CD11b and IL-4Rα by flow cytometry. While IL-4Rα surface expression was detected on unstimulated (23.4%) or IL-4/IL-13-stimulated BMMϕ from WT (17.8%), no surface expression could be detected in BMMϕ derived from IL-4Rα$^{-/-}$ mice, confirming deletion of the IL-4Rα gene (Figure 1B).

IL-4/IL-13 activation of macrophages antagonizes IFNγ induction of the inducible nitric oxide synthase (iNOS) (Gordon, 2003). In order to control their alternative activation, BMMΦ were preincubated with or without a cocktail of IL-4 and IL-13 followed by a further activation with IFN-γ and LPS. As shown in (Figure 1C), IL-4 and IL-13 preincubation was able to reduce NO production in WT mice but not in IL-4Rα$^{-/-}$ mice.

To examine therefore the activation state of BMMϕ derived from WT or IL-4Rα$^{-/-}$ mice following stimulation with IL-4, IL-13 or a cocktail of IL-4 and IL-13,

macrophages were stained for intracellular MMR or Ym-1 expression and analyzed by flow cytometry (Figure 1D). Incubation of BMMϕ from both WT and IL-4Rα$^{-/-}$ mice, with medium showed no increase in YM-1 or MMR expression However IL-4, IL-13 and IL-4/IL-13 stimulation of WT mice caused an increase in MMR (45.2%, 27.9% and 33.6% respectively) and YM1 expression (51.4%, 22.5% and 52.2% respectively)while MMR or YM-1 expression was not not upregulated in IL-4Rα$^{-/-}$ BMMϕ (Figure1D). When IL-4Rα$^{-/-}$ BMMϕ where stimulated MMR decreased from 19.9% (with medium alone) to 8.8% with IL-4, 15.6% with IL-13 and increased to 26.9% with IL-4/IL-13. YM1 (when stimulated) decreased from 21.7% with medium alone to 15.5%, with IL-4, 15.8% with IL-13 and increased to 27.5% with IL-4/IL-13.These results demonstrate that MMR and YM-1 expression are up regulated in BMMϕ upon alternative activation in an IL-4Rα dependent manner.

1.2.2 *S.mansoni* egg-induced TH2 response promote YM-1 and MMR expression on macrophages.

S.mansoni egg antigens drive the development of a strong TH2 immune response (Atochina *et al.*, 2008; Grzych *et al.*, 1991; Wilson *et al.*, 2007). To determine whether the T_H2 induced response to *S.mansoni* eggs could promote alternative activation of macrophage *in vivo* in an IL-4Rα dependent manner WT, IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice were injected intraperitoneally with *S.mansoni* eggs. Seven days following injection, peritoneal inflammatory cells were isolated and the expression of MMR and YM-1 on peritoneal macrophages (CD11b^{hi}F4/80^{hi}) was analyzed by flow cytometry. While a 24-48 hour period would be enough to assess the role of the innate immune system in driving AAMϕ activation, the seven day period/incubation allowed assessment of the role of both the innate and adaptive immune system on expression of YM-1 and MMR by macrophages.

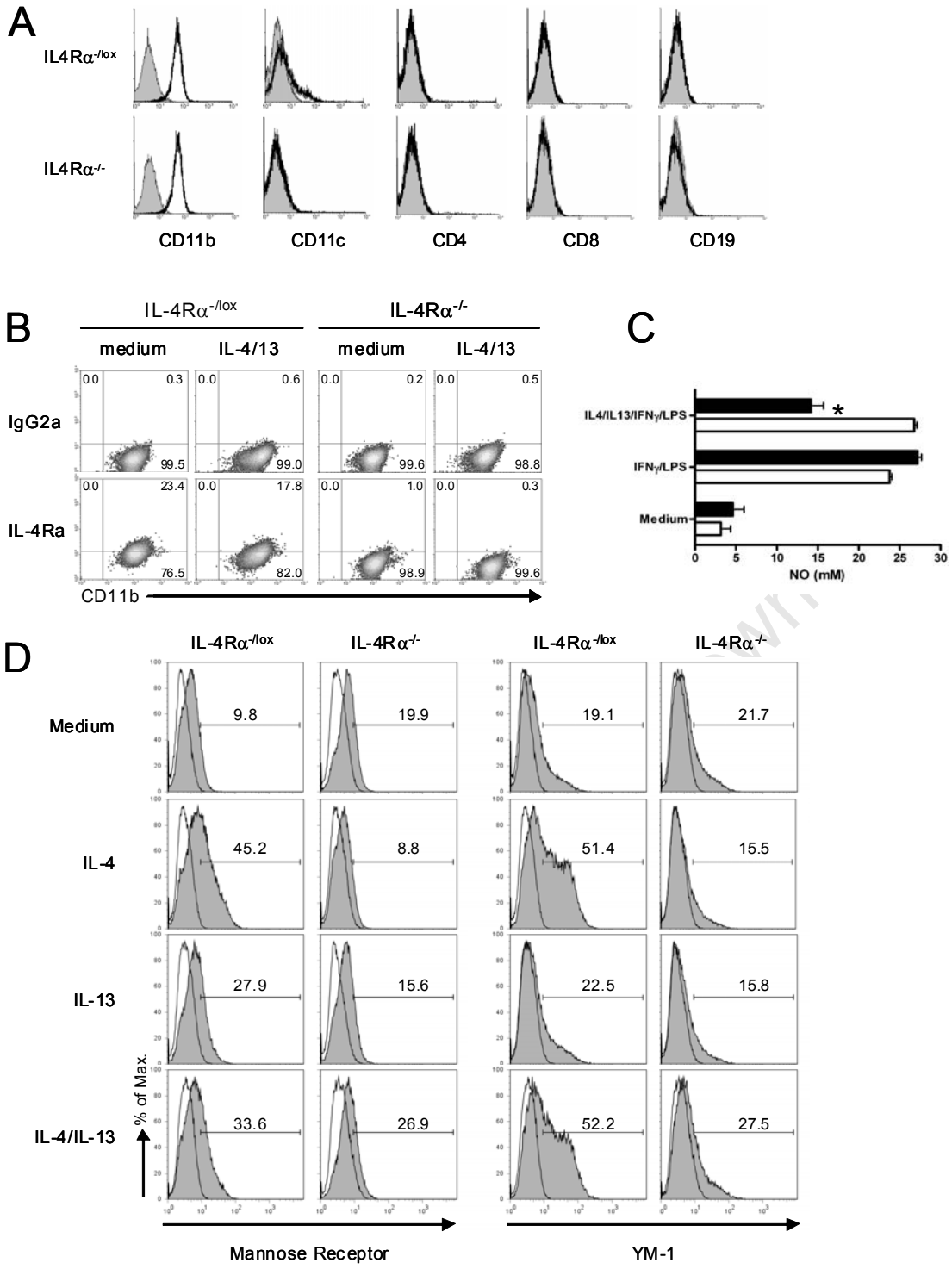


Figure. 1. IL-4 and IL-13 induced expression of mannose receptor and YM-1 in BMMϕ *in vitro*. A. BMMϕ phenotype was verified after differentiation *in vitro* and staining for CD11b, CD11c, CD4, CD8 and CD19, respectively. B. Surface IL-4Rα staining was performed on BMMϕ derived from IL-4Rα$^{-/lox}$ or IL-4Rα$^{-/-}$ following 72h incubation in presence or absence of IL-4 (10ng/ml)and IL-13 (10ng/ml). C. Intracellular mannose receptor or Ym-1 stainings were performed on BMMϕ derived from IL-4Rα$^{-/lox}$ or IL-4Rα$^{-/-}$ following 72h incubation in presence or absence of IL-4 (10ng/ml)and IL-13 (10ng/ml).

Whereas IL-4Rα$^{-/-}$ mice failed to express MMR (19%) and YM-1 (4%), WT mice had a high expression of MMR (87%) and YM-1 (80%). Surprisingly a subpopulation of peritoneal cells from LysM^{cre}IL-4Rα$^{-/lox}$ mice also expressed MMR (74%) and YM-1 (39%%) (Figure 2B).

Using this approach the result indicates that : (i) Either there is an IL-4Rα independent mechanism of inducing MMR and Ym-1 expression on macrophages (ii) or that the mechanism used to delete IL-4Rα from macrophage does not result in 100% deletion and it is this population that responds to IL-4/IL-13 induced by eggs. If the former conclusion is correct this suggests that YM-1 and MMR may be not good markers of alternative macrophage activation. However LysM^{cre}IL-4Rα$^{-/lox}$ mice infected with *S.mansoni* have increased susceptibility (Herbert *et al.*, 2004) and therefore we further investigated YM-1 and MMR expression in acute phase granuloma.

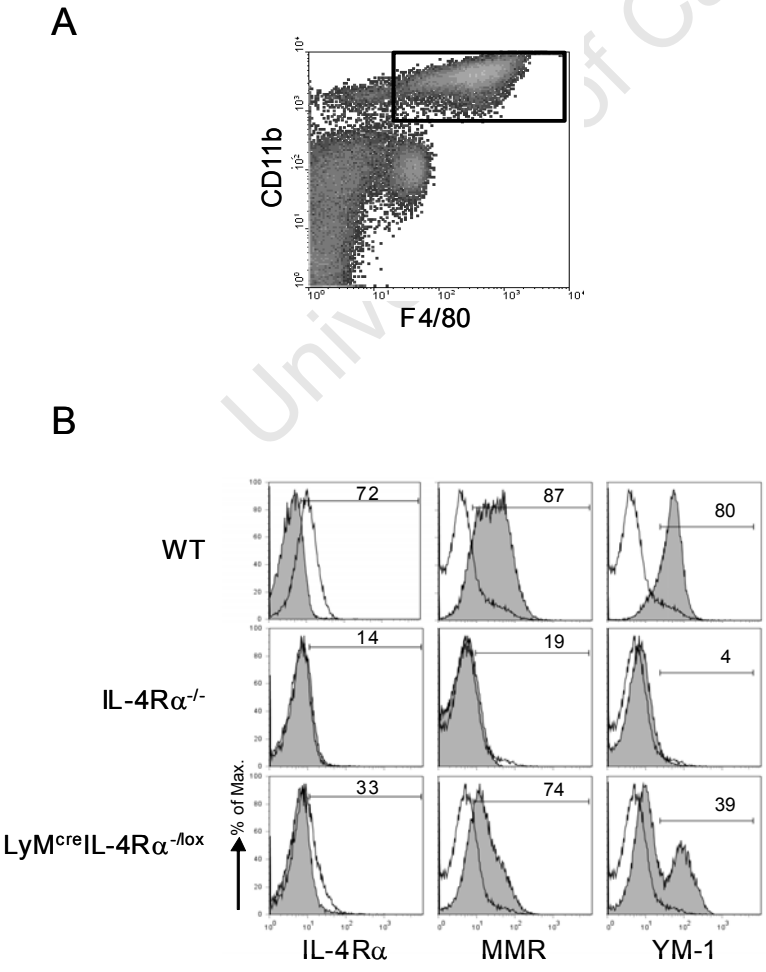


Figure. 2. *S. mansoni* egg-induced inflammation induces mannose receptor (MMR) and YM-1 expression in IL-4/13 responsive peritoneal macrophages. Seven days after egg injection, peritoneal cells were harvested and stained for F4/80 and CD11b before surface staining of MMR expression or intracellular staining for Ym-1 expression performed. A. Representative dot plot of F4/80 and CD11b double staining on peritoneal cells. Gating strategy is shown. B. Monoparametric histograms showing MMR or Ym-1 expression following gating on double positive F4/80^{hi}CD11b^{hi} cells as shown in A.

1.2.3 IL-4Rα impairment on macrophages alters the localization and/or expression of macrophage activation markers in *S.mansoni*-induced granuloma

Whereas AAMϕ were demonstrated to be essential for survival during acute schistosomiasis (Herbert *et al.*, 2004), little is known about their role played in the control of *S.mansoni*-induced granulomatous inflammation. In order to determine the effect of IL-4/IL-13 responsiveness on the activation state of macrophages during live *S.mansoni* infection we analysed and compared the expression and localization of macrophage activation markers in granuloma from WT, IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice at 8 weeks post infection. (See materials and methods for specific trouble shooting conditions)

CD11b and F4/80 are two determinants commonly used to target macrophages. However, both markers are expressed on eosinophils and macrophages (Taylor *et al.*, 2003). The specific detection of macrophages becomes consequently complex during parasite infections such as schistosomiasis. To overcome this problem, we targeted the scavenger receptor-A (CD204), which is specifically expressed on macrophages (de Villiers *et al.*, 1994; Hughes *et al.*, 1995; Tomokiyo *et al.*, 2002). We stained liver (Figure 3) and intestinal (Figure 4) tissues using immunofluorescence*****.

[NB: * *Granuloma with eggs were selected and analyzed for differential expression. All the representative pictures shown in figure 3 to 8 contain eggs surrounded by granulomatous tissue however all or part of the egg may fall out of the granuloma during sectioning, as a result some section only show partial eggs (egg shells (e.g. figure 3 panel i) with (e.g. figure 3 iii) or without mericidia (e.g. figure 3 panel X))*].

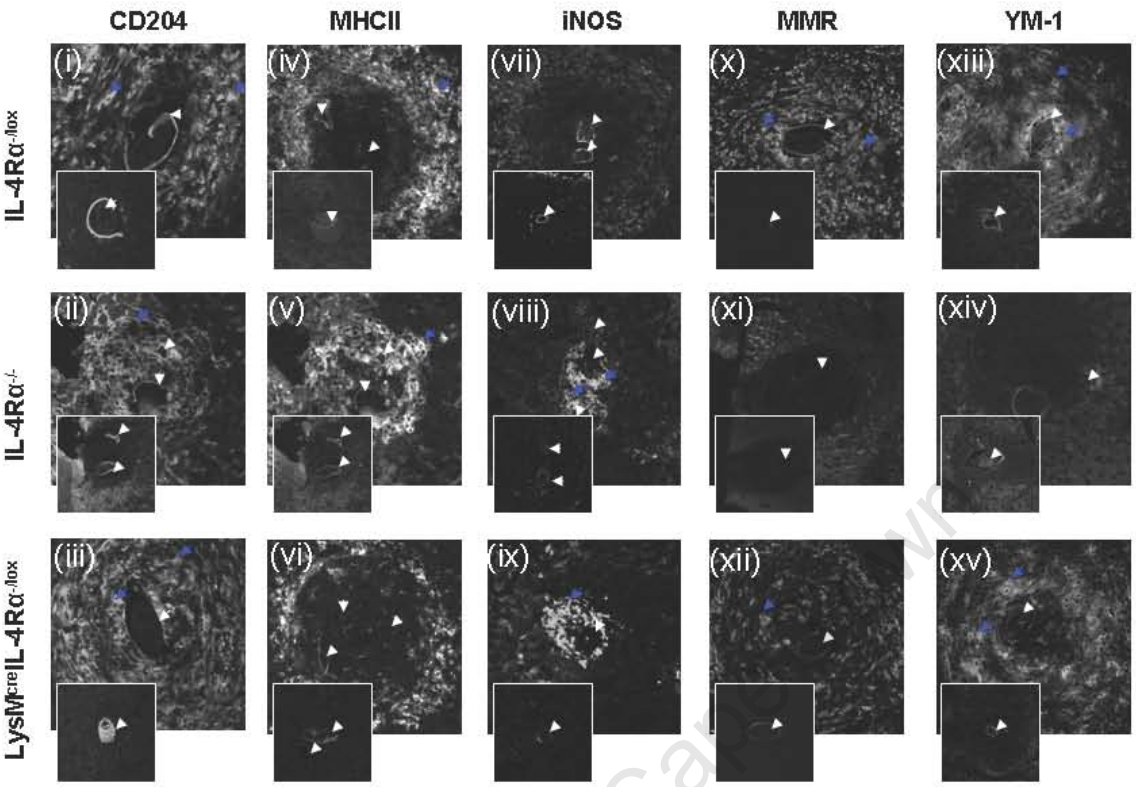


Figure. 3: Localization and/or expression of macrophage activation markers in liver granuloma during *S. mansoni* infection. Immunofluorescence staining was performed on frozen liver sections from the indicated strains at 8 weeks post-infection. Scavenger receptor II/III (CD204), Mannose receptor (MMR), (YM-1) and inducible nitric oxide synthase (iNOS). (Magnification 200X Image 3.81cm 1cm=50µm). Inserts show isotype/negative control. White arrows indicate *S.mansoni* egg, blue arrow heads indicate examples of positive staining. Representative monochromatic micrographs of more than 2 independent experiments. Approximately 10 granulomas were analyzed per experiment per group the chosen images represent more than 70% of the granuloma

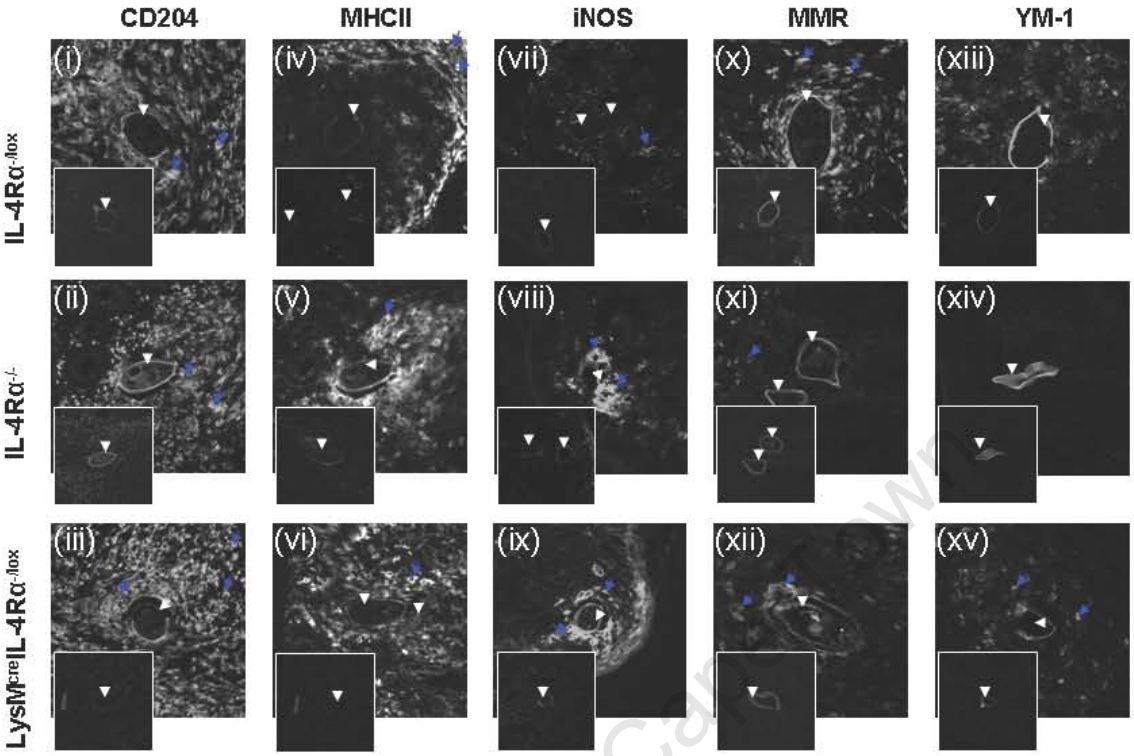


Figure. 4: Localization and/or expression of macrophage activation markers in intestinal granuloma during *S. mansoni* infection. Immunofluorescence staining was performed on frozen liver sections from the indicated strains at 8 weeks post-infection. Scavenger receptor II/III (CD204), Mannose receptor (MMR), (YM-1) and inducible nitric oxide synthase (iNOS). (Magnification 200X image is 3.81cm 1cm=50μm). Inserts show isotype/negative control. White arrows indicate *S.mansoni* eggs, blue arrow heads indicate examples of positive staining. Representative monochromatic micrographs of more than 2 independent experiments. Approximately 10 granulomas were analyzed per experiment per group the chosen images represent more than 70% of the granuloma.

In both tissues, granuloma expressed CD204 in all groups (Figure 3 and 4, panels i-iii). The CD204 positive cells were scattered throughout the granuloma (examples are indicated with small blue arrow heads). Granuloma from IL-4Rα$^{-/-}$ mice had fewer CD204 positive cells around the eggs compared to WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice in the liver (Figure 3, panel ii). The latter observation result from the fact that IL-4Rα$^{-/-}$ mice make significantly smaller granuloma (Herbert *et al.*, 2004; Jankovic *et al.*, 1999). Nevertheless the results here show that macrophages are present in the granuloma of WT, LysM^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice.

As MHC-II is upregulated in activated macrophages, immunostaining was performed to detect the latter determinant in liver and intestinal sections (Figure 3 and 4, panels iv-vi). In WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice, MHC-II positive cells were mostly localized at the periphery of the liver granuloma (Figure 3, panels iv and vi). In IL-4Rα$^{-/-}$ granuloma sections however, MHC-II positive cells were condensed around the egg (Figure 3, panel v). In the intestine the level of staining was lower for all groups, the trend in localization of MHC-II positive cells in WT and IL-4Rα$^{-/-}$ mice was similar to the liver sections (Figure 4, panels iv-vi). In LysM^{cre}IL-4Rα$^{-/lox}$ mice intestine sections however the positive cells were not localized at the periphery but were more scattered in the granuloma.

Since macrophages are present in the granulomas of all groups. We next aimed to determine the macrophage activation state inside the granuloma in relation to IL-4Rα expression using the main marker of classically activated macrophages iNOS (Gordon, 2003). High levels of iNOS were detected in granuloma macrophages in liver and intestine from IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 3 and 4, panels viii and ix). In contrast, very low iNOS expression was detected in granuloma macrophages from WT mice in both tissues (Figure 3 and 4, panel vii). This indicated that WT mice contain very few classically activated macrophages while classically activated macrophages are present in higher numbers in the granulomas of IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$.

As previously published (Linehan *et al.*, 2003), granuloma macrophages from WT expressed high levels of MMR in liver and intestine (Figure 3 and 4, panel x). However, granuloma macrophages from IL-4Rα$^{-/-}$ mice did not express MMR in both tissues (Figure 3 and 4, panel xi). Surprisingly, granuloma macrophages from LysM^{cre}IL-4Rα$^{-/lox}$ expressed MMR in the liver and intestine at similar level compared to WT mice (Figure 3, panels x and xii). These results suggest that MMR is expressed independently of macrophage IL-4Rα signaling.

In order to further investigate AAMϕ in granuloma we performed YM-1 immunostaining on liver and intestinal granuloma. YM-1 was expressed in granulomas from WT mice while its expression could not be detected in IL-4Rα$^{-/-}$ mice (Figure 3 and 4, panels xii and xiv). Again YM-1 however was detected at relatively high levels in the granuloma of LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 3 and 4, panel xv). These results support that MMR and YM-1 may not be specific for AAMϕ.

1.2.4 Further analysis of MMR and YM-1 expression in granuloma.

In order to further define surface expression of MMR and YM-1, we examined the co-expression of macrophage activation markers in *S. mansoni* induced granuloma. Co-stainings of MMR and YM-1 or YM-1 and iNOS were performed on liver (Figure 5 or Figure 7) and intestine sections (Figure 6 or Figure 8) from WT, IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice at 8 weeks post infection. Cells present on the periphery of the granuloma co-expressed MMR and YM-1 in liver and intestinal granuloma from WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5 and 6). However, a discrete population at the area closely surrounding the eggs expressed high levels of YM-1 in granuloma from WT mice (Figure 5 and 6,) but co-expressed low levels of MMR (Figure 5 and 6). In contrast no YM-1^{+} population could be detected at the area closely surrounding the eggs in granuloma from IL-4Rα$^{-/-}$ or LysM^{cre}IL-4Rα$^{-/lox}$ mice (Figure 5 and 6).

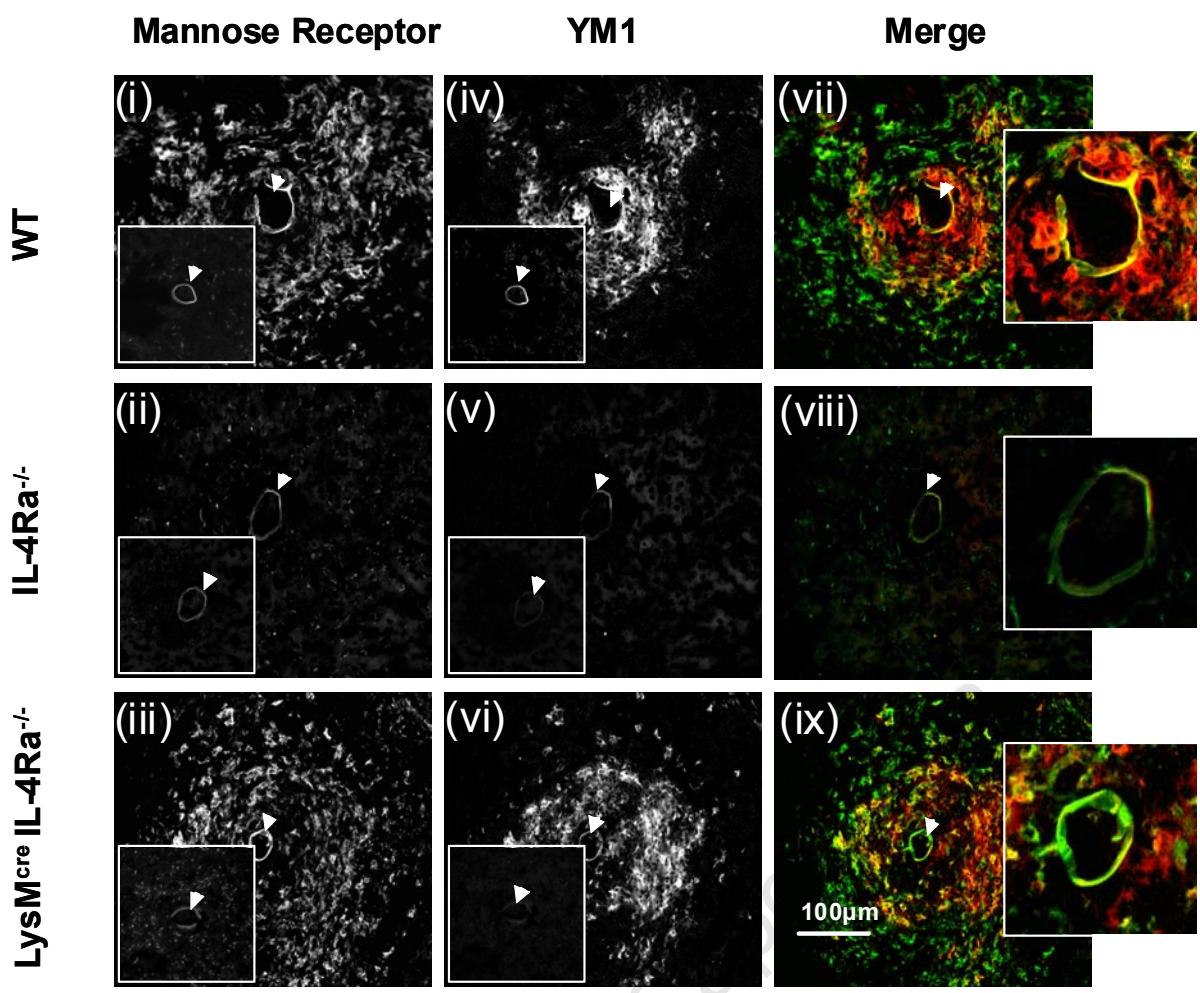


Figure 5 : Co-localisation of YM1 and Mannose receptor expressing cells close to the egg in liver granuloma is IL-4Rα$^{-/-}$ and Macrophage IL-4Rα$^{-/-}$ dependant. Immunofluorescence staining was performed on frozen sections of the liver granulomas from the indicated strains 8 weeks PI. Panel i-iii MMR and Panel iv-vi YM1 with an insert of their respective negative controls. Panel vii-ix Merged images of MMR was labeled FITC (green) and YM1 was labeled with PE (red). (Magnification 200X) Insert on merged images shows magnification of the area around the egg. White arrow heads indicate eggs. Data is representative of 2 experiments. 10 granulomas were analyzed per experiment per group the chosen images represent more than 70% of the granuloma.

High levels of iNOS expression were however detected at the close surrounding of eggs in granuloma from IL-4Rα$^{-/-}$ or LysM^{cre}IL-4Rα$^{-/lox}$ mice in both the liver and intestine (Figure 7 and 8, panel v-vi) but no/low expression of iNOS was detected in granuloma from WT mice (Figure 7 and 8,). The results suggest that IL-4Rα is necessary for the expression of YM-1 at the close surrounding area of the egg.

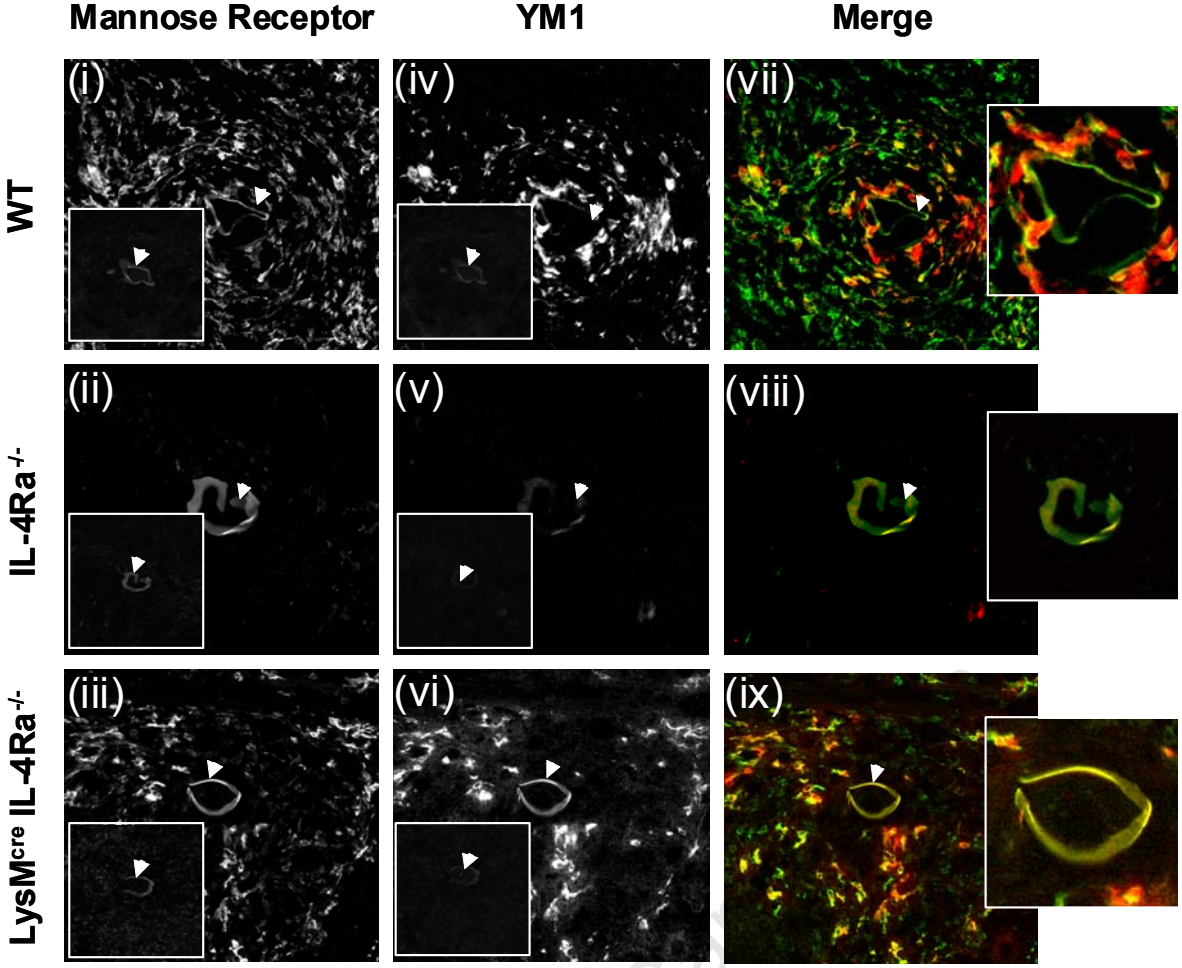


Figure 6: Co-localization of YM1 and Mannose receptor expressing cells close to the egg in intestinal granuloma is IL-4Ra$^{-/-}$ and Macrophage IL-4Ra$^{-/-}$ dependant. Immunofluorescence staining was performed on frozen sections of the intestinal granulomas from the indicated strains. Panel i-iii MMR and Panel iv-vi YM-1 with an insert of their respective negative controls. Panel vii-ix Merged images of MMR was labeled FITC (green) and YM1 was labeled with PE (red). (Magnification 200X) Insert on merged images shows magnification of the area around the egg. White arrow heads indicate eggs. Data is representative of 2 experiments. 10 granulomas were analyzed per experiment per group the chosen images represent more than 70% of the granuloma.

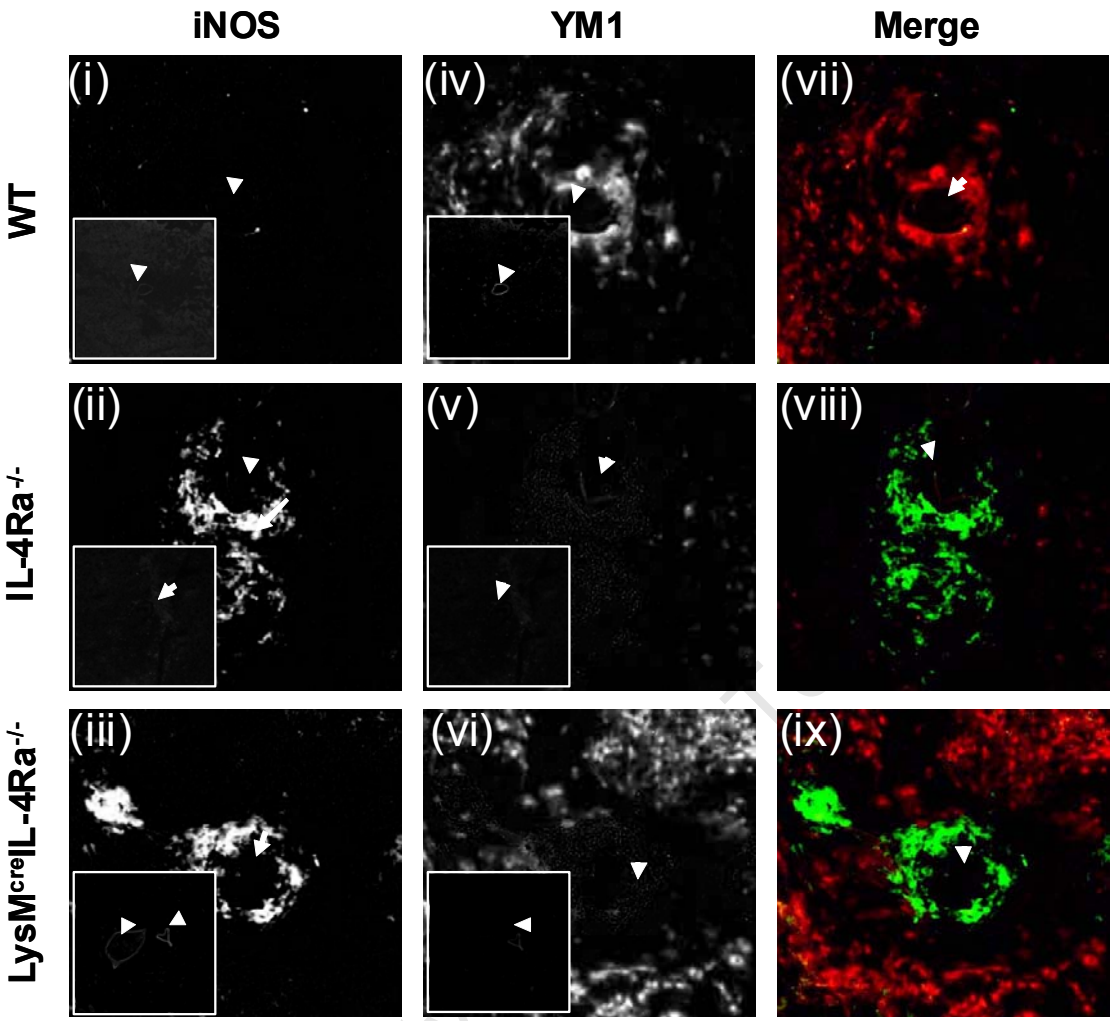


Figure 7 : Alternative versus classical macrophage activation in liver granuloma during *S. mansoni* infection. Immunofluorescence staining was performed on frozen liver sections from the indicated strains at 8 weeks post-infection. Panel i-iii iNOS and panel iv-vi YM1 with an insert of their respective negative controls. Panel vii-ix merged images of YM1 was labeled with PE (red) and iNOS was labeled with FITC (green) (Magnification 200X).White arrows indicate eggs. Representative micrographs of 2 experiments are shown. Approximately 10 granulomas were analyzed per experiment per group the chosen images represent more than 70% of the granuloma.

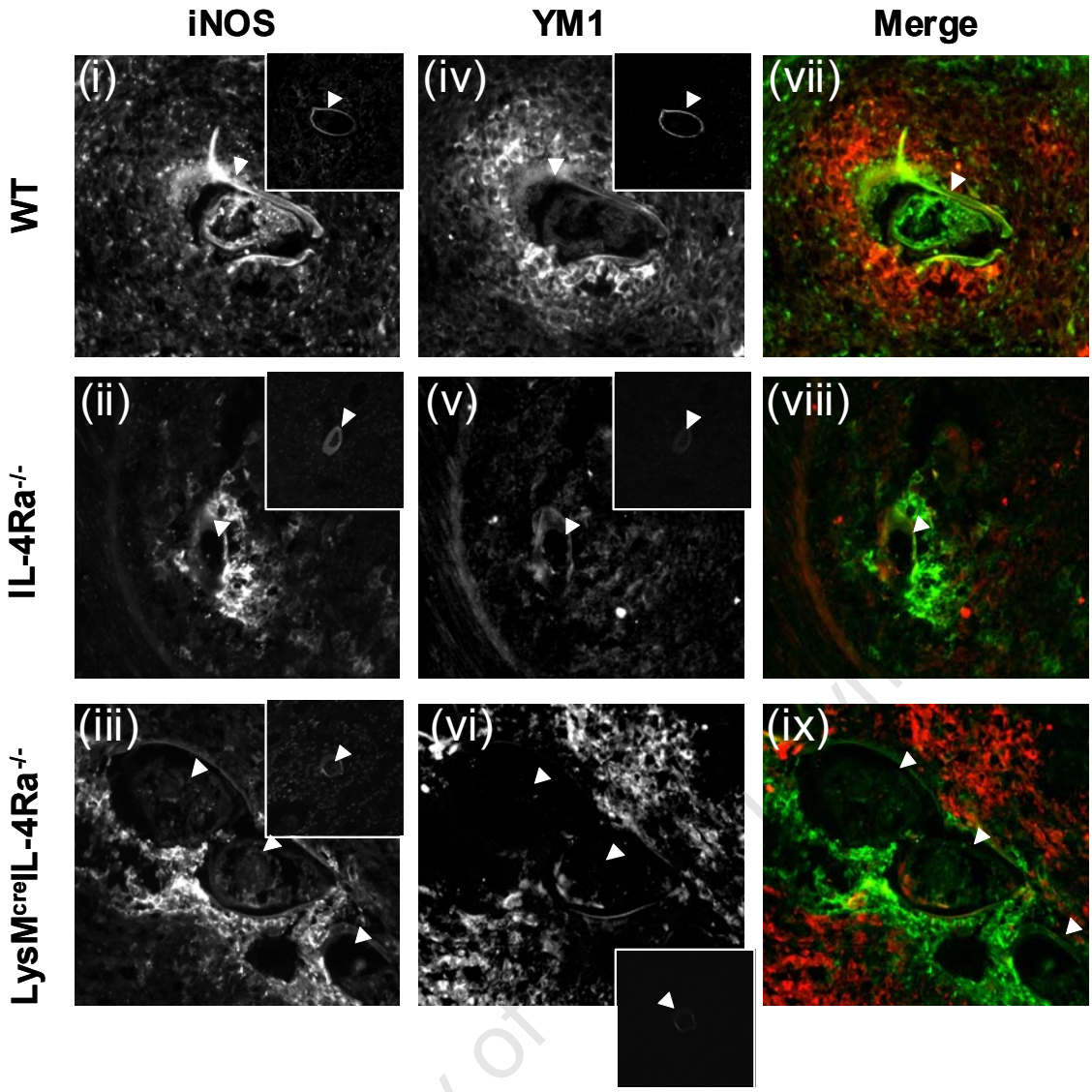


Figure 8 : Alternative versus classical macrophage activation in intestinal granuloma during *S. mansoni* infection. Immunofluorescence staining was performed on frozen gut sections from the indicated strains at 8 weeks post-infection. Panel i-iii iNOS and panel iv-vi YM1 with an insert of their respective negative controls. Panel vii-ix merged images of YM1 was labeled with PE (red) and iNOS was labeled with FITC (green) (Magnification 200X).White arrows indicate eggs. Representative micrographs of 2 experiments are shown. Approximately 10 granulomas were analyzed per experiment per group the chosen images represent more than 70% of the granuloma.

1.2.5 IL-4Rα impairment does not affect B220^{+} cell recruitment in granuloma during *S.mansoni* infection

To determine whether the activation state of IL-4Rα disrupted macrophages had an effect on B-cell recruitment, we stained sections for B220 expression. In the liver B220^{+} cells were similarly found at the periphery of granuloma from WT and LysM^{cre}IL-4Rα$^{-/flox}$ mice (Figure 9, panels i and iii). Although IL-4Rα$^{-/-}$ had smaller granulomas the staining pattern was similar to the other groups (Figure 9, panels ii). In the gut however, B220^{+} cells were found at the periphery of granulomas

from WT mice but scattered in the granuloma from IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/flox}$ mice.

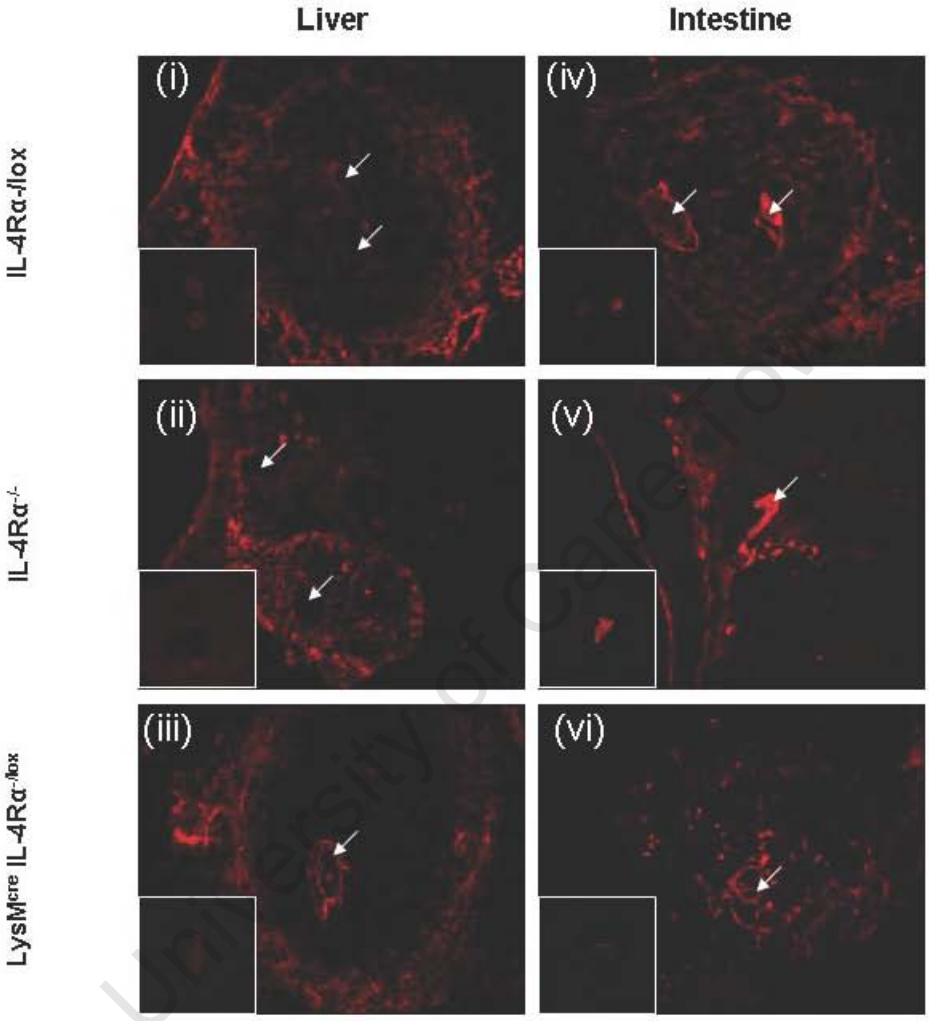


Figure. 9. B220 cell accumulation in liver and intestinal granuloma during *S. mansoni* infection. B220 labeled with PE (red) immunofluorescence staining of liver and intestinal frozen section at 8 weeks post-innfection. Insert shows the iso-type/negative control (Magnification 200x) Approximately 10 granulomas were analyzed per experiment per group the chosen images represent more than 70% of the granuloma. White arrow= *S.mansoni* egg

1.2.6 IL-4/IL-13 responsive macrophages reduce T cells numbers in the granuloma

To examine the effect of AAMϕ on T cells we stained sections to detect CD3 expressing cells. While all groups had positive cells, the gene deficient mice had more CD3 positive cells ($CD3^+$) than the WT mice in the liver and intestinal granuloma (Figure 10A). In the intestine $CD3^+$ appeared mainly in the villi but were also present in the granuloma of WT and gene deficient mice (Figure 10). In the liver of WT and $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ these positive cells seemed to accumulate predominantly at the periphery of the granuloma while in the $IL\text{-}4R\alpha^{-/-}$ positive cells were densely packed around the egg (Figure 10).

To determine the level of CD3 positive cell infiltration/proliferation, the edge of the granulomas were marked (Figure 10A dashed line) and the numbers of positive cells were counted in the selected region. Quantification of the $CD3^+$ cells in the granulomas indicated that $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ and $IL\text{-}4R\alpha^{-/-}$ had significantly higher $CD3^+$ cells in the liver than WT mice (Figure 10C). The $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice also had significantly more $CD3^+$ cell in the intestine (Figure 10B). Due to the poor granuloma formation in the ileum quantification was difficult, particularly in the $IL\text{-}4R\alpha^{-/-}$ mice were the edge of the granuloma could not be defined (ND). The results indicate that IL-4 responsive macrophages may regulate directly or indirectly the recruitment and/or proliferation of T cells at site of inflammation.

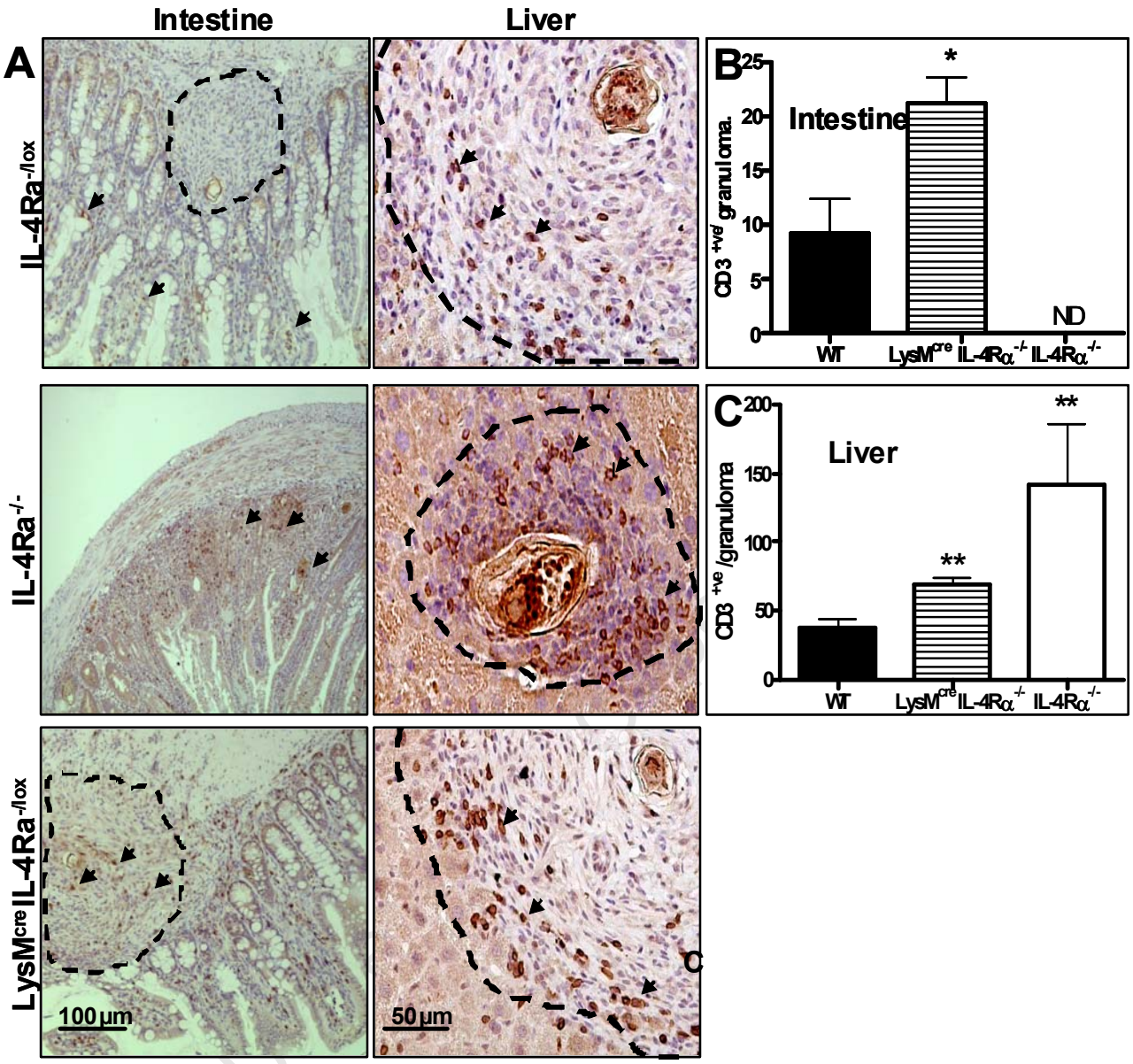


Figure. 10. Granuloma associated T cells are more numerous in the liver and intestine of IL-4Ra$^{-/-}$ and LysM^{cre} IL-4Ra$^{-/lox}$ mice compared to WT mice. Immunohistochemical staining for CD3^{+} cells on paraffin embedded intestinal (Magnification 100x) and liver sections (Magnification 200x 1cm=50µm). at 8 weeks post-infection. DAB chromogen was used to detect positively stained cells (seen as dark brown dots) and counterstained with Haematoxylin (blue). Black arrows indicate examples of positive staining. Dashed line illustrates the demarcation of granulomas used for quantification of CD3+ cells per granuloma. B and C Quantification of CD3 positive cells per granuloma in the intestine (B) and Liver (C). * $p<0.05$,**$p<0.01$, ND the granuloma area was not definable in the intestine for IL-4Ra$^{-/-}$ mice. Representative of 2 experiment n>3 mice per group, data is the average of more than 10 granulomas ± SEM.

1.3 DISCUSSION

During *S.mansoni* infection eggs trapped in the tissue drive T_H2 dependent immunopathology that is characterized by granuloma formation (Doughty *et al.*, 1984). Activated in response to T_H2 cytokines, IL-4Rα responsive macrophages are essential for host survival however their exact role is not known (Herbert *et al.*, 2004). These macrophages were said to be alternatively activated. Alternatively activated macrophages express IL-4Rα i.e. they respond to IL-4 and IL-13 and express a wide range of markers including Arginase 1, YM-1, MMR, Fizz-1. (Gordon, 2003; Loke *et al.*, 2002; Raes *et al.*, 2002).

In the present study we confirmed that MMR and YM-1 expression depends on a IL-4/IL-13 triggered IL-4Rα response on bone marrow derived macrophages. However when $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ mice that lack IL-4Rα on macrophages specifically, were stimulated in the peritoneum with *S. mansoni* eggs which induce the TH2 response, we observed a subpopulation of MMR and YM-1 expressing macrophages. This was supported by the fact that granulomas of $LysM^{cre}IL\text{-}4R\alpha^{-/flox}$ also expressed MMR and YM-1 similar to WT mice. We concluded that MMR and YM-1 were not specific markers of an alternative macrophages activation state. Interestingly however, a population of cells that was highly positive for YM-1 localized at the area closely surrounding the eggs was noticed in the WT mice and this was IL-4Rα dependent. Finally, we showed that while $B220^+$ cells localized in the granuloma independently of IL-4Rα expression on macrophages, T cells recruitement and/or proliferation in the granuloma depend on IL-4/IL-13 responsive macrophages

A common feature in liver and intestinal granulomas is the abundance of macrophages (Weinstock & Boros, 1981). During schistosomiasis immature granuloma contain approximately 30 to 50% eosinophils, intermediate granulomas contain a mix of eosinophilic granulocytes, macrophages and few lymphocytes, while mature granulomas contain predominantly macrophages that

become large and pigmented (epithelioid hystiocytes) (Moreels *et al.*, 2001; Swartz *et al.*, 2006). Weinstock et al. (1983) showed that liver granuloma are composed of 30% macrophages while ileal granuloma contains 75% of macrophages. By using macrophage scavenger receptor-A (CD204) staining we confirmed here that macrophages are dispersed throughout the granuloma in the liver and intestine in an IL-4Rα independent manner. The staining also revealed that the macrophages are in close association with the egg (de Villiers *et al.*, 1994; Tomokiyo *et al.*, 2002). These macrophages express functional markers that are used to distinguish them from one another. The markers used in this study (MMR and YM-1) may be stimulated by both the innate and adaptive immune factors but can also be stimulated directly by bacterial factors such as endotoxin or factors secreted by helminthes such as chitin (Reese *et al.*, 2007). *S.mansoni* however does not contain chitin. Instead, Atochina et al 2008 recently showed that an immunomodulatory glycan (LNFPIII) up regulates expression of YM-1 and Arginase 1 on macrophages in an IL-4Rα independent manner. LNFPIII contains a Lewis X trisaccharide which is also found in *S. mansoni* egg. This suggests that eggs possess the potential to directly induce YM-1 and Arginase 1 expression.

Arginase-1 is a definitive marker of alternative activation however using the immunohistochemical/fluorescence approach in this study the presence of arginase was not clear, as endogenous arginase in the tissue made background staining very high especially in the liver (Gordon, 2003)(Loke *et al.*, 2007). We therefore used YM-1 and MMR as markers of IL-4/IL-13 responsiveness on macrophages (Gordon, 2003) (Linehan *et al.*, 2003; Loke *et al.*, 2007; Welch *et al.*, 2002). Upon stimulation with IL-4/IL-13 or *S.mansoni* eggs or during granuloma formation MMR and YM-1 expression was found in WT mice but this was abrogated in global IL-4Rα$^{-/-}$ mice (Raes *et al.*, 2005; Welch *et al.*, 2002). Therefore in contrast to Atochina et al. we suggest that IL-4/IL-13 signaling during live infection is required to some extent for YM-1 production. This contradiction may be explained by the difference in experimental approach.

Atochina *et al.* (2008) used RT-PCR (mRNA production) data while we used protein expression to determine YM-1 production. It may be that YM-1 is up regulated on the mRNA level in our studies but this is not evident at the protein level. In support of an IL-4Rα independent mechanism however IL-10 together with IL-2 has also been shown to induce AAMϕ markers (Edwards *et al.*, 2006). Furthermore our data also shows that YM-1 and MMR were both produced in $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ granuloma and peritoneal macrophages suggesting that another mechanism regulates the expression of these markers specifically on macrophages. Interestingly $IL\text{-}4R\alpha^{-/-}$ mice have been shown to be deficient in IL-10 production while $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice produce high levels of IL-10 and therefore IL-10 may play a role in the macrophage-IL-4Rα independent expression of alternative markers (Balic *et al.*, 2006; Herbert *et al.*, 2004; Hoffmann *et al.*, 2000). It will be interesting in future to examine YM-1 and Arginase1 mRNA expression to determine if the protein expression correlates with the mRNA production during live infection.

Since YM-1 and MMR are expressed possibly by an IL-4Rα independent manner (and certainly in a macrophage-specific-IL-4Rα independent manner) the results also suggested that YM-1 and MMR are not good markers IL-4/IL-13 responsive macrophages. However since we do not directly show deletion of IL-4Rα on granuloma macrophages in infected $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice, it could also be argued that IL-4Rα is still present on cells in the granuloma, resulting in YM-1 and MMR expression.

Despite this concern however iNOS staining indicated that classically activated macrophages were present at the core of the granulomas in $IL\text{-}4R\alpha^{-/-}$ and $LysM^{cre}IL\text{-}4R\alpha^{-/-}$ mice, suggesting that IL-4/IL-13 non-responsive cells must be present in the granulomas of $LysM^{cre}IL\text{-}4R\alpha^{-/-}$ mice. Interestingly in the WT mice although they did not have this classical (iNOS+) population a second observed population of granuloma macrophages was found. This population was highly YM-1 positive while it expressed lower levels of MMR. This population was found

at the area closely surrounding the eggs in both liver and intestine. This population was absent in granuloma from IL-4Rα$^{-/-}$ mice and was similarly absent in granuloma from LysM^{cre}IL-4Rα$^{-/lox}$ mice. The induction of the highly positive YM-1 population in the granuloma and its localization inside the granuloma of WT mice suggests a dependency on the IL-4 responsiveness by macrophages. Consequently, we suggest that the YM-1 positive macrophage population localizing at the close surrounding of eggs represents an IL-4/IL-13 responsive activation state.

Beside their role in tissue repair, AAMϕ were involved in immunosuppression (Flores Villanueva *et al.*, 1994; Loke *et al.*, 2000) . IL-4Rα$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$ mice had higher numbers of T cells in liver and intestinal granuloma. AAMϕ induced during schistosomiasis could therefore play a role to regulate inflammation by suppressing T cell recruitment and/or proliferation. It is still not clear by what mechanism AAMϕ suppress T cell proliferation. Smith et al. (2004) showed that *S.mansoni* stimulate the expression of co-stimulatory molecules such as a programmed cell death ligand (PD-L1) and ICOS on macrophages (Smith *et al.*, 2004). It is strongly suggested that binding of PD-L1 and ICOS to their respective receptors on the T cell is suppress the inflammation. Interestingly these molecules are upregulated as earlily as 4 weeks post infection, suggesting that it is the worms that drive T cell anergy, also, IFNγ is responsible for induction of PD-L1 while IL-4 is responsible for PD-L2 (Loke & Allison, 2003). However PD-L2 only peaked in production 12weeks post infection and therefore may not explain the IL-4Rα dependant suppression of inflammation that is shown here and therefore another mechanism may be responsible. Following on the work by Smith et al. (2004) Colley et al. (2005) showed that PD-L2 is upregulated early during *S.mansoni* infection on B220 negative dendritic cells (peaking at 7 to 9 weeks post infection) which may suggest a IL-4Rα dependent mechanism (Colley *et al.*, 2005). This would therefore explain the increased proliferation of T cells in the liver and intestine of IL-4Rα deficient mice during the acute phase, however this may also point to yet another mechanism since LysM^{cre}IL-4Rα$^{-/lox}$

mice still have greater number of T cells compared to wild type mice. Here we therefore also suggest that immunomodulation may be associated with localization of YM1 and MMR positive producing cells.

While these results together indicate that MMR and YM-1 expression on alternative macrophages is somewhat controversial. The use of MMR and in particular YM-1 has highlighted a possibly important population of cells in the granulomas of WT mice. Although YM-1 was described as a chitinase-like protein, it lacks any chitinase activity (Chang *et al.*, 2001; Sun *et al.*, 2001). However, a recent study described its role in digestion of glycosaminoglycan and might therefore play a role in egg killing and/or antigen processing (Harbord *et al.*, 2002). It has also previously been shown that YM-1 encapsulates pathogens (Nair *et al.*, 2003). It is clear from this study that this is also the case with *S.mansoni* eggs. It may therefore be suggested that this encapsulation may serve to protect the host from proinflammatory and possibly harmful factors released by eggs thus suppressing inflammation.

These findings also highlight a reason why there is such a massive defect in IL-4Rα$^{-/-}$ mice compared to LysM^{cre}IL-4Rα$^{-/lox}$ mice. In LysM^{cre}IL-4Rα$^{-/lox}$ mice other IL-4/IL-13 responsive cells are clearly able to partially compensate for the difference seen between these mice groups. This is demonstrated by the fact that LysM^{cre}IL-4Rα$^{-/lox}$ mice are still able to form granuloma and as published by Herbert etal. (2004) have still produce TH2 cytokines and type 2 antibodies. Therefore while IL-4Rα is essential on macrophages for survival other IL-4/IL-13 responsive cells are also necessary during schistosomiasis.

Together the data presented above provide unique insight into the organization of the granuloma in terms of macrophage markers. The proposed organization of macrophages in granuloma from WT or LysM^{cre}IL-4Rα$^{-/lox}$ mice is presented in figure 11. Immunohistological staining is poorly quantifiable however from the staining we were able to observe the presence, absents and localization of AAMϕ

markers/receptors. Figure 11 shows the most dominant observation in granuloma. Mannose receptor was expressed from the core to the periphery of the granuloma in both WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice. YM-1 was found at the core of WT granuloma but was only found in the area just inside the periphery of LysM^{cre}IL-4Rα$^{-/lox}$ mice. In this same region there was a stronger co-localisation of MMR and YM-1 in WT and LysM^{cre}IL-4Rα$^{-/lox}$ mice. Lastly the B220 and MHCII both co-localised at the periphery of the granuloma. It could be suggested that at this region antigens transported from the core of the granuloma are processed and presented at the periphery of the granuloma.

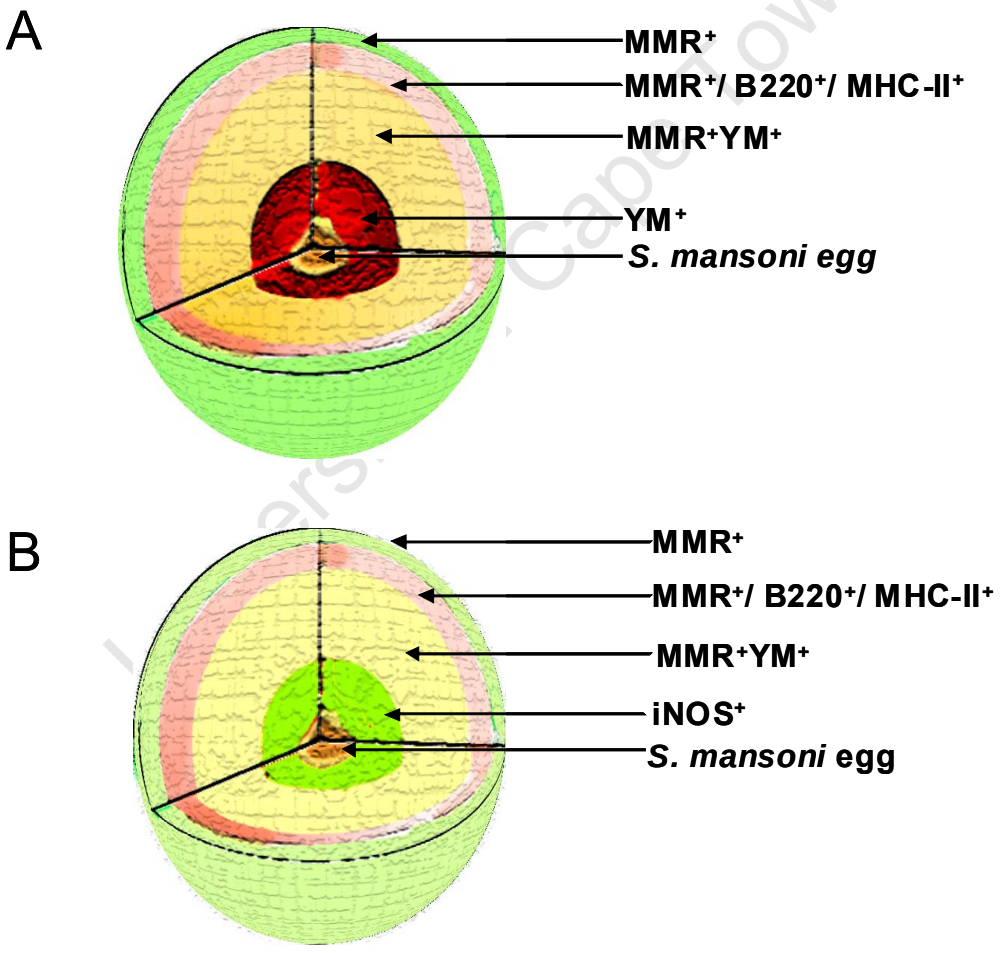


Figure 11: Granuloma in the presence and absence of IL-4/IL-13 responsive macrophages. Schematic diagram based on immunoflourescent staining. WT (A) LysM^{cre}IL-4Rα$^{-/lox}$ (B). Schistosoma mansoni eggs are found at the core of the granuloma and surrounded predominantly by MMR positive (MMR+) macrophages. At the periphery of the granuloma B220, MHCII and MMR positive cells are found in both groups. Closer to the egg double positive YM1+MMR+ cells were found in both groups. At the core of WT granuloma were predominantly YM-1 positive cells. At the core of LysM^{cre}IL-4Rα$^{-/lox}$ mice macrophages were predominantly iNOS positive cells. NB: + means that the area is highly positive for the particular marker.

Here macrophages may be alternatively activated. AAMϕ migrate to the core were they mop up apoptotic cells and factors released by eggs to reduce inflammation, as well as secrete factors involved in wound healing namely YM-1 and fibronectin which is a building block for collagen(Gratchev *et al.*, 2001). From this study and others it has become apparent that YM-1 and MMR expression are not clear indicators of IL-4/IL13 responsive macrophages. Their expression may be modulated by the parasite, by the duration of infection, by the adaptive and innate immune response. However IL-4/IL-13 does regulate iNOS expression and therefore iNOS is confirmed as reliable a negative indicator of AAMϕ. Finally these results indicate that macrophage IL-4Rα is involved in localization of YM-1 close to the eggs. Future studies should focus of determining the significance of this localisation.

CHAPTER 2

CHAPTER 2

Smooth muscle cell IL-4Rα deficient mice show increased susceptibility to *Schistosoma mansoni*

2.1. INTRODUCTION

Schistosomiasis, caused by the helminth *Schistosoma mansoni (S. mansoni),* is a major human tropical disease (Pearce & MacDonald, 2002). *S. mansoni* cercariae from infected water enter the host through the pores in the skin and migrate to the mesenteric vessels where they differentiate and mature into egg producing adult worms (Elliott, 1996). Eggs are released in the blood vessels and follow the stream to reach the mesenteric capillaries before they migrate through the intestinal tissue and are excreted with the host's faeces. The mechanism by which these eggs pass through the mucosa remains unknown, however since immune compromised humans and mice fail to clear eggs, egg transit is considered an immune dependant process (Doenhoff, 1997).

Before egg deposition by adult worms, the immune response to *S. mansoni* is a weak Type 1 immune response during the first 4 weeks post-infection (p.i.) (Pearce *et al.*, 2004; Pearce & MacDonald, 2002). Eggs deposited in the tissue however, drive a shift to strong Type 2 immune response with a subsequent production of T_H2 cytokines, including interleukin (IL)-4 and IL-13. IL-4 and IL-13 mediate their activity by binding to the IL-4 receptor alpha (IL-4Rα), found on both haematopoietic and non-haematopoietic cells and induce a signalling cascade by the Jak/Stat6 pathway (Andrews *et al.*, 2002; Finkelman *et al.*, 2000; McKenzie *et al.*, 1999; Metwali *et al.*, 2002; Murata *et al.*, 1999; Pearce *et al.*, 2004). Previously we reported that IL-4Rα deficient mice (IL-4Rα$^{-/-}$) fail to excrete *S. mansoni* eggs as efficiently as wild type mice (WT) resulting in severe intestinal inflammation (Fallon *et al.*, 2000a; Herbert *et al.*, 2004; Hoffmann *et al.*, 2000).

Herbert et al. (2004) showed that mice lacking IL-4Rα on macrophages and neutrophils ($LysM^{cre}$IL-$4R\alpha^{-/flox}$), similarly to IL-$4R\alpha^{-/-}$ mice, fail to excrete eggs efficiently and succumb to infection before WT mice (Herbert *et al.*, 2004). The $LysM^{cre}$IL-$4R\alpha^{-/flox}$ mice had severe intestinal pathology, hepatocyte damage and increased endotoxin (LPS) levels similar to global IL-$4R\alpha^{-/-}$ mice. The mechanism(s) by which these IL-4/IL-13 responsive cells regulate inflammation and mediate egg expulsion remain unknown. Nevertheless, while the necessity of IL-4/IL-13 responsiveness on macrophages has clearly been demonstrated, direct evidence for a role of IL-4/IL-13 responsiveness on non-haematopoietic cells has not been described during schistosomiasis (Herbert *et al.*, 2004; Hoffmann *et al.*, 2000; Leeto *et al.*, 2006; Pearce *et al.*, 1996).

The non-haematopoietic cell, smooth muscle cell, has been implicated in a number of helminth associated disease. Smooth muscle is non-striated muscle which is found within: arteries and veins, the bladder, uterus, reproductive organs , the respiratory and gastrointestinal tracts and the iris. In these organs the chief function of smooth muscle is contraction which is predominantly controlled by the parasympathetic nervous system. When neurotransmitters are released by the postsynaptic ganglion they bind to receptors on the effecter cell. These receptors are intergral to the membrane, thus conformational changes induced upon binding the neurotransmitter causes permeability changes in the membrane. Ions for example calcium, potassium and sodium rush in or out of the cell which in the case of smooth muscle results in a depolarization of the cell and the propagation of an action potential. Since smooth muscle cells (smc's) are joined together by gap junctions which allows action potentials to be transferred from one cell to another. These signals result in the relaxation and/or contraction of the smooth muscle and therefore the organ they form part of.

Neurotransmitters are divided into four classes: Class I (only acetylcholine) Class II (the hormones such as epinephrine), Class III (the amino acids such as glycine) and Class IV (the peptide such as tachykinin substances P) (Dittman & Kaplan, 2008; Guyton, 1987).

In smooth muscle acetylcholine is an important excitatory neutrotransmitter but may also have inhibitory effects. When acetylcholine is released it binds to the cholinergic receptors: muscarinic acetylcholine receptors (mAchR) and nicotinic acetylcholine receptors (nAchR)(Furness, 2000; Goyal & Hirano, 1996). mAchR are G-protein coupled receptors which are divided into 5 distinct G subtypes: M1 to M5. M3 is the main mediator of contraction in smooth muscle while M1 receptor induces relaxation pathways on SMC's (PW Stengel 2002 and N Struckmann 2003)(Ehlert *et al.*, 1999). In comparison to nAchR (coupled to ion channels), mAchR as a result of G-protein coupling are slow acting receptors (Caulfield 1993 and Birdsall 1998)(Ishii & Kurachi, 2006). nAchR are therefore thought to initiate muscle contraction while mAchR results in sustained increase in contractility. In addition to being found on SMC's mAchR are also found on epithelial cells and inflammatory cells: macrophages and T cells. Here they are involved in release of glandular secretions such as mucous and hormones, cytokine release and degranulation of inflammatory cells (Ishii & Kurachi, 2006). Another important neurotransmitter is the tachykinin substances P, which falls under the class IV/ neuropeptide group of neurotransmitters. Substance P binds to the neurokinin 1 (NK 1) receptor which mediates excitatory signals. NK1 is also a G protein coupled receptor, found on the muscular plexus, interstitial cells of Cajal (which regulate peristalsis), in neurons and myoid cells of the villi (Faussone-Pellegrini & Vannucchi, 2006). Together these neurotrasmitters are released to mediate contraction of the tissue they are found in.

In the digestive tract, smooth muscle contracts in a peristaltic fashion, which forces digested material through the gastrointestinal tract. It is this peristaltic contraction of smooth muscle that has been implicated in a number of helminth

diseases. It may therefore be suggested that the immune system directly/indirectly controls this system to eliminate intestinal pathogens. In particular the Th2 immune response which is induced by most helminths, for example *Nippostongylus .brasiliensis* and *T .spiralis*, has been shown to induce hypercontracility of smooth muscle (Akiho *et al.*, 2002; Horsnell *et al.*, 2007). The Th2 cytokines (IL-4/IL-13) and regulatory factors such as TGFβ have been linked to increase mAchR and ligand expression/release and therefore stimulate increased contractility (Akiho *et al.*, 2007).

In vivo and in vitro studies have shown that Stat-6 deficient smooth muscle cells from helminth infected mice have reduced response to contraction stimulants (Akiho *et al.*, 2002; Khan *et al.*, 2001). Since Stat-6 is the downstream signalling molecule of IL-4Rα, the results suggest that IL-4/IL-13 responsiveness is required for increased contractility. Recently Horsnell et al. (2007) showed that during *Nippostongylus .brasiliensis* infection, mice lacking IL-4Rα on smooth muscle cells (SM-MHC^{cre}IL-4Rα$^{-/flox}$) have delayed goblet cell hyperplasia and worm expulsion as well as reduced expression of M3 (Horsnell *et al.*, 2007). It was therefore suggested that reduced expression of the receptor results in decreased contractility which results in abrogated *N. brasiliensis* worm expulsion (Horsnell *et al.*, 2007). Interstingly NK1 expression has also been shown to be altered during *N.brasiliensis* infection (Faussone-Pellegrini *et al.*, 2002). The T_H2 response induced by *S. mansoni* eggs has also been shown to mediate hypercontractility (Bogers *et al.*, 2000; El Zawawy *et al.*, 2006; Moreels *et al.*, 2001; Moreels *et al.*, 2004). An increased proliferation of smooth muscle cells during the acute phase of schistosomiasis has been observed (Moreels *et al.*, 2001) and results in functional changes in the muscularis during the chronic schistosomiasis. The latter observation may suggest that effects on SMC contractility will be seen in the chronic phase (Bogers *et al.*, 2000; Moreels *et al.*, 2001; Moreels *et al.*, 2004). The major gastrointestinal complications that stem from changes in gastrointestinal motility (diarrhoea, dysentery, anaemia)

however, occur during the acute phase of schistosomiasis (Gryseels, 1992; van der Werf *et al.*, 2002).

In this manuscript we aimed to study the role of IL-4/IL-13 responsiveness by SMC during schistosomiasis. Infection studies were performed by using SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice and comparing them to wild type BALB/c (WT) mice. We analysed mortality, weight loss, egg burden and the ability to these mice to induce a successful T_H2 response. Finally we examined the effect of specific IL-4Rα deletion in SMC on Nk1 and muscarinic receptor M3 expression. Together these results demonstrate that while IL-4/IL-13 responsiveness by SMC is not necessary for induction of a successful Type 2 response during schistosomiasis, it is necessary for mediating the egg excretion from the tissue, morbidity and survival. We suggest that IL-4Rα signalling in SMC during *S.mansoni* infection plays a role during the peak of egg production, allowing efficient migration of eggs from mesenteric vessels to the intestinal lumen.

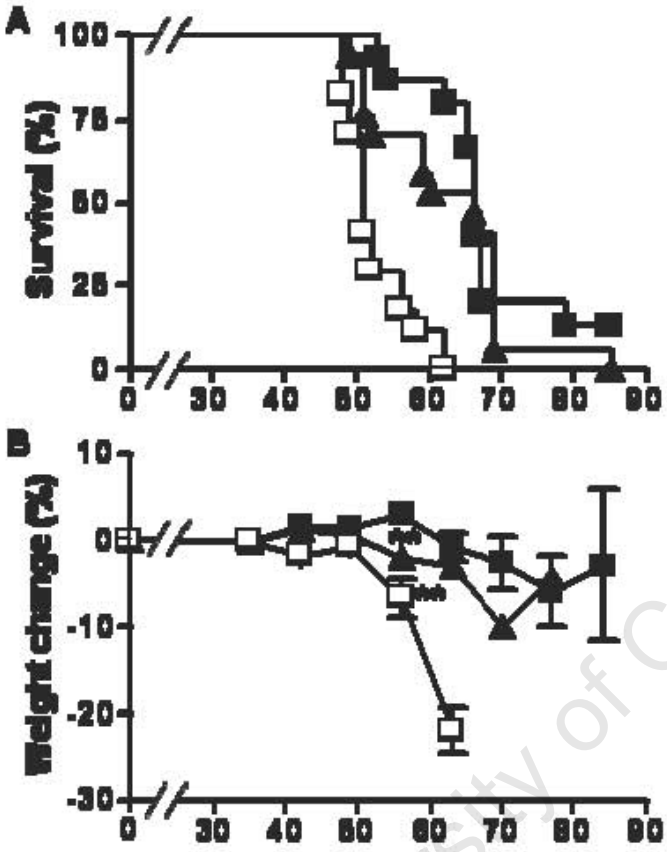
2.2 RESULTS

2.2.1 SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice increased mortality and morbidity during *S. mansoni* infection

In order to test whether the role of IL-4Rα responsiveness by SMC during schistosomiasis could vary depending on parasite burden, we performed experiments in an infectious dose kinetic. WT, SM-MHC^{cre} IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 70, 80 or 100 *S. mansoni* cercariae. As shown in Fig. 1A, SM-MHC^{cre} IL-4Rα$^{-/lox}$ showed increased mortality during the first 8 weeks/56 days of infection compared to WT controls but were not as susceptible as global IL-4Rα$^{-/-}$ mice. This was irrespective of the infection dose as shown in the table insert (Figure1A). By day 56 at all three infectious doses, IL-4Rα$^{-/-}$ had the highest mortality: (71% when infected with 70 cercariae compared to 85% mortality when infected with 100 cercariae), WT mice had the lowest mortality, (0% when infected with 70 cercariae compared to 30% mortality when infected with 100 cercariae) while SM-MHC^{cre} IL-4Rα$^{-/lox}$ had 10% mortality infected with 70 cercariae compared to 38% mortality infected with 100 cercariae. However after 56 days susceptibility between the WT and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice was similar. These results indicate that deletion of IL-4Rα specifically on smooth muscle results in an intermediate and transient increase in susceptibility during the first 56 days post infection

To determine the effect of IL-4Rα deletion on SMC on the morbidity during schistosomiasis the body weights were recorded. Similarly to the mortality, IL-4Rα$^{-/-}$ mice lost weight rapidly after 42 days p.i. (Fig. 1B). Some IL-4Rα$^{-/-}$ mice lost more than 20% of their original body weight before death. The SM-MHC^{cre} IL-4Rα$^{-/lox}$ and WT mice began to lose weight from 42 days p.i., albeit at a slower rate than the IL-4Rα$^{-/-}$ mice (Fig. 1B). At day 56 p.i., the SM-MHC^{cre} IL-4Rα$^{-/fox}$ mice had a significantly greater weight loss compared to WT mice. However after

day 56 the percentage weight loss was similar in the SM-MHC^{cre} IL-4Rα$^{-/lox}$ and WT mice.



Mouse	70 Cerc.	80 Cerc.	100 Cerc.
WT	0%	12%	30%
SM-MHC^{cre}IL-4Rα^{lox}	10%	38%	38%
IL-4Rα$^{-/-}$	71 %	87%	85%

Figure1

Effect of IL-4Rα deletion on SMC during *E. muricoli* infection

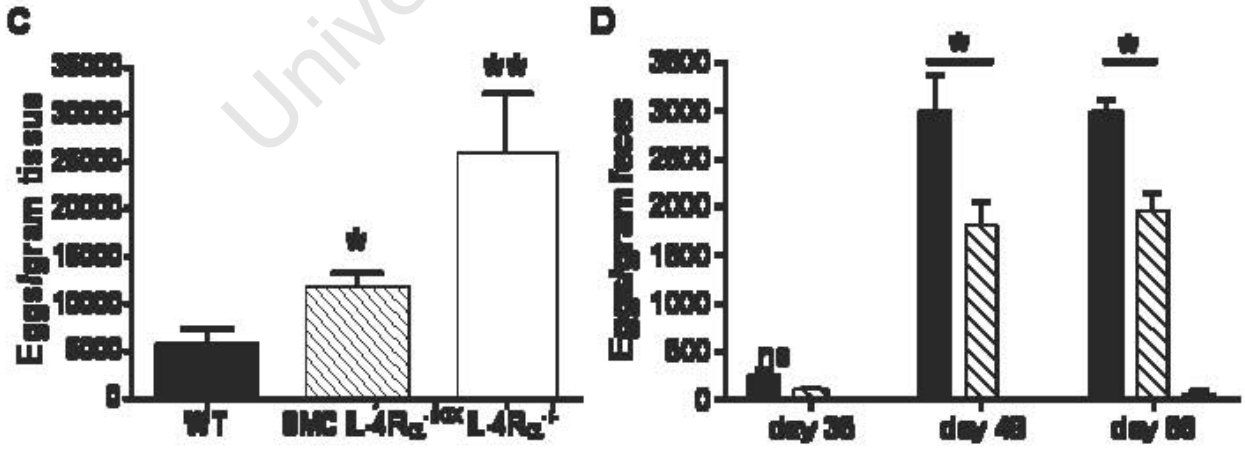
A) *Survival.* WT (square) n=13, SM-MHC^{cre}IL-4Rα^{lox} (triangle) n=15 and IL-4Rα-/- (open square) n=12 (data are pooled from three experiments. Table insert shows the mortality at day 56 for three different dose of infection (70, 80 or 100 cercaria).

B) *Morbidity:* *E. muricoli* infected with 80 cercaria, mice were weighed weekly. *WT (square) n=12,* SMC IL-4Rα-/- (triangle) n=12 and IL-4Rα-/- (open square) n=6. Data (means ± SEM) are representative of 3 independent experiments.

** *P<0.001 (significantly different from WT mice).*

C) Three egg burden: Intestinal egg burden was assessed for WT (black bar) (n=6), SM-MHCcre IL-4Rα-flox (locked) (n=5) and IL-4Rα-/- (white bar) (n=5) mice 8 weeks p.i. Tissue was incubated in KOH overnight before egg count. Data (means ± SEM) are representative of 3 experiments. Infected with approximately 80 cercaria *P<0.05, **P<0.001 *(significantly different from WT mice)*

D) Fecal egg output: Fecal pellets were obtained from WT (black bar), SM-MHCcre IL-4Rα-flox (locked bar) and IL-4Rα-/- (open bar) mice at day 35, day 49 and day 56 p.i. before egg count. Infected with 100 cercaria, Data representative of 2 experiments, are means ± SEM. *P<0.05 *(significantly different from WT mice).*



The results described above demonstrate an increased susceptibility, with largest differences in weight loss at day 56 in SM-MHC^{cre} IL-4Rα$^{-/lox\ -}$ mice compared to WT mice, which interestingly coincides with the peak of egg production (week 8/day 56 post infection) and subsequent T_H2 immune response (Pearce & MacDonald, 2002).

2.2.2 IL-4Rα responsiveness by SMC promotes egg migration and excretion.

IL-4/IL-13 $^{-/-}$ and IL-4Rα$^{-/-}$ mice have abrogated egg transit through the intestine (Herbert *et al.*, 2004). To determine if the role of IL-4Rα signaling in SMC in egg transit, egg burden was examined in the intestine at day 42 (beginning of egg accumulation and expulsion) and day 56 (peak egg production and expulsion) post infection (p.i.).

At 6 weeks p.i., no difference in egg burden could be detected in the intestine of all groups. However, intestines from IL-4Rα$^{-/-}$ and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice contained significantly more eggs compared to WT mice at day 56 p.i. (Fig. 1C), .

In order to determine that the higher egg burden in IL-4Rα$^{-/-}$ and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice result from a deficiency in egg migration and/or excretion, fecal egg samples were obtained from the infected WT, SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/-}$ mice at day 35, 49 and 56 p.i. At week five/day 35 very few eggs were detected in all the groups and no significant difference was found. At day 49 and 56 p.i., SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice excreted significantly lower numbers of eggs compared to WT mice (Fig. 1D). In IL-4R α$^{-/-}$ infected mice eggs were almost undetectable in the feces at all the time points indicated (Fig 1D).

Altogether, the results describe above suggest that the increased susceptibility at week 8 is associated with abrogated egg transit and output.

2.2.3 Increased egg burden does not result in gut damage in SM-MHC^{cre} IL-4Rα$^{-/lox}$.

To determine whether increased tissue egg burden in SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice resulted in a similar intestinal pathology observed in IL-4Rα$^{-/-}$ mice and therefore explain the increase in susceptibility (Herbert *et al.*, 2004), we examined intestinal histopathology and LPS levels in SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice in comparison to WT and IL-4Rα$^{-/-}$ mice.

Small intestine and colon sections were fixed, cut and H&E stained to examine histopathology and immunopathology (granuloma formation) Compared to naïve mice, infected WT small-intestine changes in morphology were characterized by broadened and shortening of villi due to granuloma formation (Figure 2A see yellow circles), diffuse inflammatory infiltrate (Figure 2A see irregular blue line), and dilation of lacteal spaces (Figure 2A, indicated by stars), Interestingly, WT mice showed localised thickening of the muscularis propria in regions were granuloma formed (Fig. 2A panels (i-ii) area marked MM). In contrast, IL-4Rα$^{-/-}$ mice had severe enteritis (Figure 2 A irregular blue line), congested capillaries (Figure 2 A panel vi white double headed arrows) and larger lacteal spaces (Figure 2 A panel vi red star). Infected SM-MHC^{cre} IL-4Rα$^{-/lox}$ had similar morphological changes compared to WT mice, without significant difference in thickening of the muscularis propria (Fig. 2A panels iii). Analysis of the large intestine of infected mice similarly revealed no differences in histopathology between the groups (Figure 2A panel vi-viii). With regards to granuloma formation: whereas no discrete mature granuloma could be detected in intestine of infected IL-4Rα$^{-/-}$ mice, no difference between granuloma distribution and size could be detected between WT and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice (Fig. 2E).

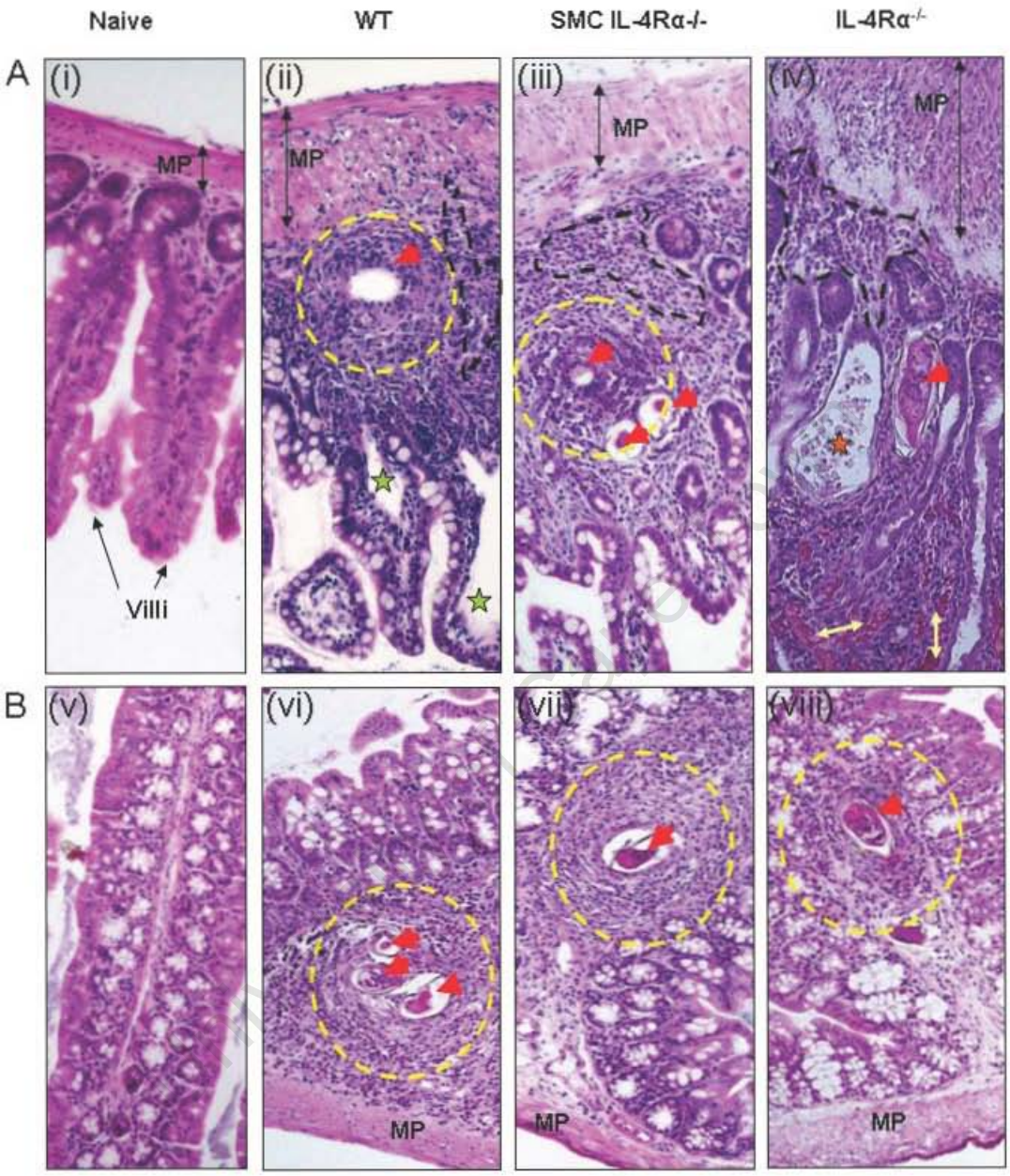


Figure 2
Histopathology of *S.mansoni* infected IL-4Rα^{flox} (WT), SM-MHC^{cre}IL-4Rα^{flox} and IL-4Rα$^{-/-}$ mice. Granuloma formation and tissue morphology (A): Small intestine and **(B)** large intestine sections (H&E, 100x). (MP, muscularis propria, dilated lacteal spaces indicated ★ with cellular infiltrate or ★ without cellular infiltrate, ↔; congestion; ⟵; *S.mansoni* eggs in the tissue and yellow circle demarcates the granuloma, dashed black line demarcates enteritis.)

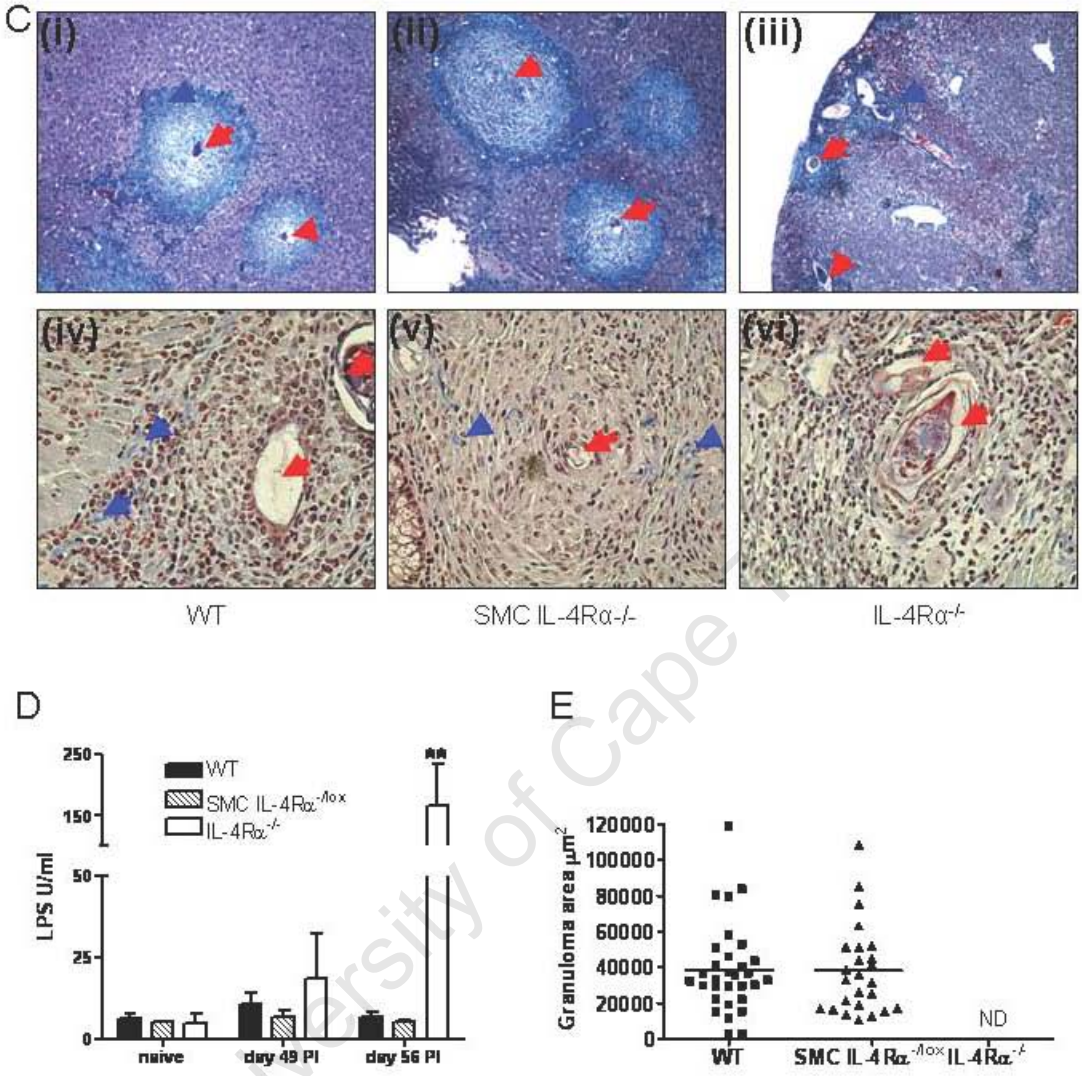


Figure 2 continued:
Fibrosis (C): CAB,hematoxylin stain of: panel (i-iii) Liver tissue sections (100x Magnification) and panel (iv-vi) Ileum tissue sections. Collagen appears blue (400x) NB: red arrows indicate egg, blue arrow indicates positive stain.
Sepsis (D): Endotoxin units in sera from WT (black bar), SM-MHC^{cre}IL-4Rα$^{-/flox}$ (hashed bars) and IL-4Rα$^{-/-}$ (open bars) mice. Data are means ± SEM of 2 similar experiments, n=4 mice per group. ** p<0.01 compared to WT
Granuloma formation (E): Ileum tissue was obtained from WT (squares), SM-MHC^{cre}IL-4Rα$^{-/lox}$ (triangles) and IL-4Rα$^{-/-}$ (open bars) mice, 8 weeks p.i.. Granuloma size was determined from H&E stained histological sections using computerised morph-metric analysis program (NIS elements by NIKON). ND, not determined.

Fibrosis in the liver, intestine and blood vessels during *S.mansoni* infection is often linked to the chronic phase pathology but increased fibrosis during the acute phase have been associated with decreased susceptibility (Herbert *et al.*, 2004). To determine if there were any differences in acute phase fibrosis between these groups we stained liver and intestinal sections with CAB (see methods). Collagen deposition was similar in liver tissue in WT and SM-MHC^{cre} IL-4Rα$^{-/lox}$, while deposition was lower in the IL-4Rα$^{-/-}$, which is possible due to the smaller granuloma size (Figure2C panel i-iii). Collagen deposition in intestinal granuloma compared to the liver granuloma however, was minimal in WT, SM-MHC^{cre} IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice indicating that fibrosis or collagen does not play a role in susceptibility during acute phase schistosomiasis (Fig. 2 C panel iv-vi).

Supporting the observation that infected SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice did not have increased intestinal inflammation compared to WT mice, levels of serum endotoxin at day 56 p.i. were similar between SM-MHC^{cre} IL-4Rα$^{-/lox}$ and WT mice (Fig. 2D). In contrast, high levels of endotoxin were measured in the serum of infected IL-4Rα$^{-/-}$ mice. Altogether, these results demonstrate that increased susceptibility of SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice can not be explained by increased intestinal inflammation.

2.2.4 Intact immune response in SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice.

The shifting of a weak T_H1 to a dominant T_H2 response which peaks at day 56 p.i. is essential for survival during acute schistosomiasis.(Pearce & MacDonald, 2002; Wynn *et al.*, 1994; Wynn *et al.*, 1997)

To determine whether the immune response elicited in SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice during *S.mansoni* infection was comparable to WT mice, we analysed antigen-specific cytokine response by MLN cells and splenocytes at week 8 p.i.. SEA-restimulated MLN cells and splenocytes from WT and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice showed equivalent levels IL-4 and IL-13 cytokines, while complete IL-4Rα$^{-/-}$ mice had significantly lower levels of IL-4 and IL-13 (Fig. 3A and B). Whereas IL-

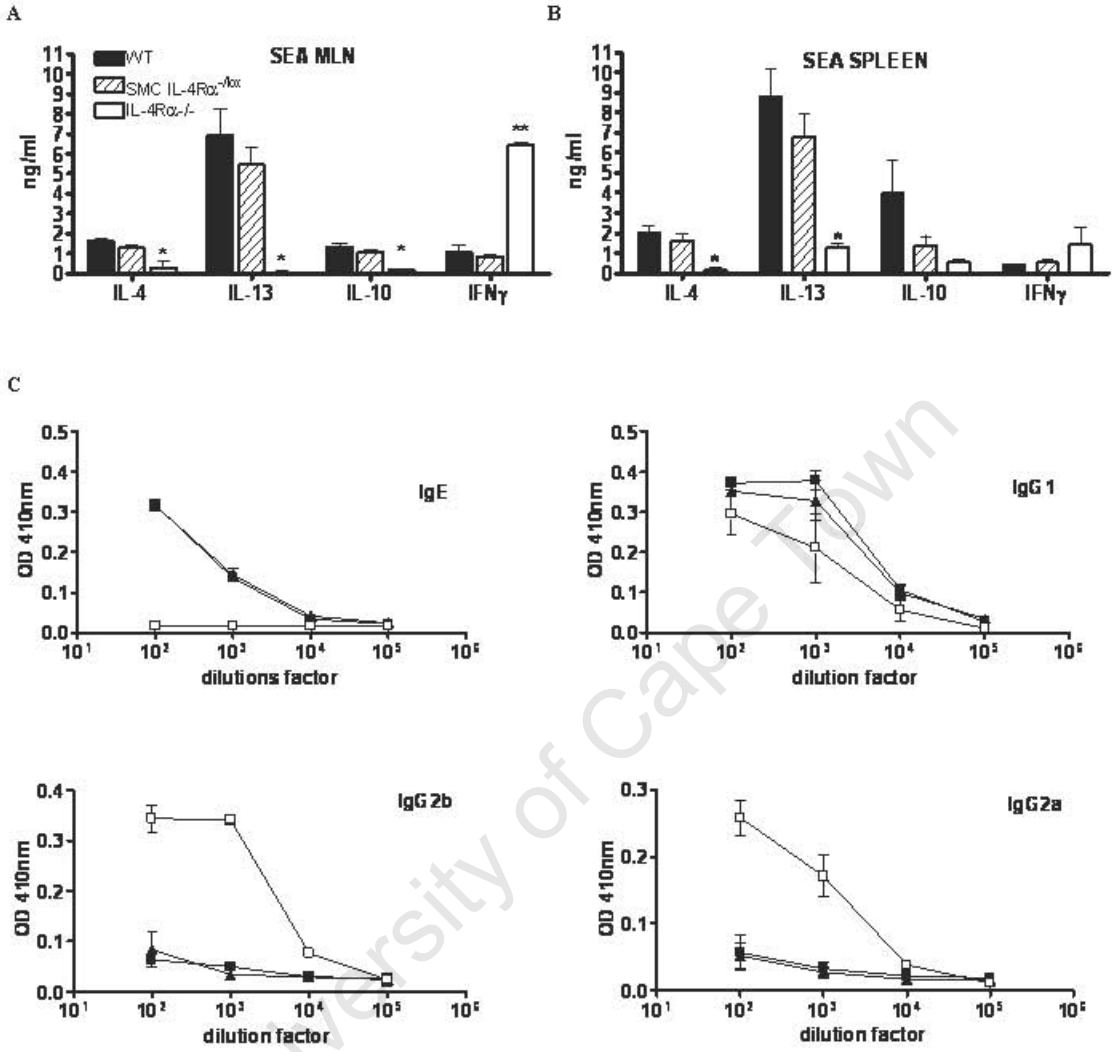


Figure 3
Intact immune response in SM-MHC^{cre}IL-4Rα$^{-/lox}$
A) Antigen specific cytokine production : MLN cells from WT (black bars), SM-MHC^{cre}IL-4Rα$^{-/lox}$ (hashed bars) and IL-4Rα-/- (open bars) were restimulated with SEA and supernatants were analysed for cytokine production. Data are means ± SEM, representative of 3 experiments, n=4 mice per group.*P<0.05, **P<0.01
B) Antigen specific cytokine production: Splenocytes from WT (black bars), SM-MHC^{cre}IL-4Rα$^{-/lox}$ (hashed bars) and IL-4Rα-/- (open bars) were restimulated with SEA and supernatants were analysed for cytokine production. Data are means ± SEM, representative of 3 experiments, n=4 mice per group. *P<0.05
C) B cell responses: Antigen-specific antibody production (IgG1, IgG2a, IgG2b) and total IgE antibody production were determined from sera of WT (black squares), SM-MHC^{cre}IL-4Rα$^{-/lox}$ (triangles) and IL-4Rα$^{-/-}$ (open squares), 8 weeks p.i.. Data are means ± SEM, n=4 mice per group. Data is representative of 3 independent experiments.

4Rα$^{-/-}$ mice had higher IFNγ levels in both spleen and MLN cells, SM-MHC^{cre}-IL-4Rα$^{-/lox}$ and WT mice had lower levels of IFNγ (Fig. 3A and B).
Similarly, antigen specific IgG1 and total IgE levels were equivalent in SM-MHC^{cre} IL-4Rα$^{-/flox}$ and WT mice but higher than the global IL-4Rα$^{-/-}$ mice (Fig. 3C). As expected antigen specific IgG2a and IgG2b production were higher IL-4Rα$^{-/-}$ mice compared to the SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice (Fig. 3C).

The results describe above indicate that IL-4/IL-13 responsiveness of SMC is not required during *S.mansoni* infection to drive the T_H2 response.

2.2.5 *S. mansoni* infection downregulates muscarinic receptor expression.

The results obtained in this study demonstrate that IL-4Rα signalling in SMC plays a role in the migration and/or excretion of eggs though the intestinal tissue. However, the observed increased egg burden does not result in increased intestinal inflammation. To determine whether the actual contractility of SMC is affected by IL-4Rα responsiveness and perhaps mediates egg expulsion, we examined the expression of the receptors responsible for SMC contractility, M3 and NK1 in the intestine of WT, IL-4Rα$^{-/-}$ and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice.

As shown in Fig. 4, naïve mice had similar levels of M3 receptor expression while NK1 was significantly higher in IL-4Rα$^{-/-}$ mice compared to WT mice (Fig. 4). Interestingly infection caused a significant decrease in M3 and NK1 receptor expression in all groups (Fig. 4). Unexpectedly the M3 expression was high in the IL-4Rα$^{-/-}$ mice compared to the WT and SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice 8 weeks p.i..

Together these results show that IL-4Rα responsiveness by smooth muscle cells is not essential for induction of the immune response or maintenance of tissue morphology during acute schistosomiasis. However disruption of responsiveness to IL-4/IL-13 by SMC result in increased egg retention and a transient increase in weight loss and mortality.

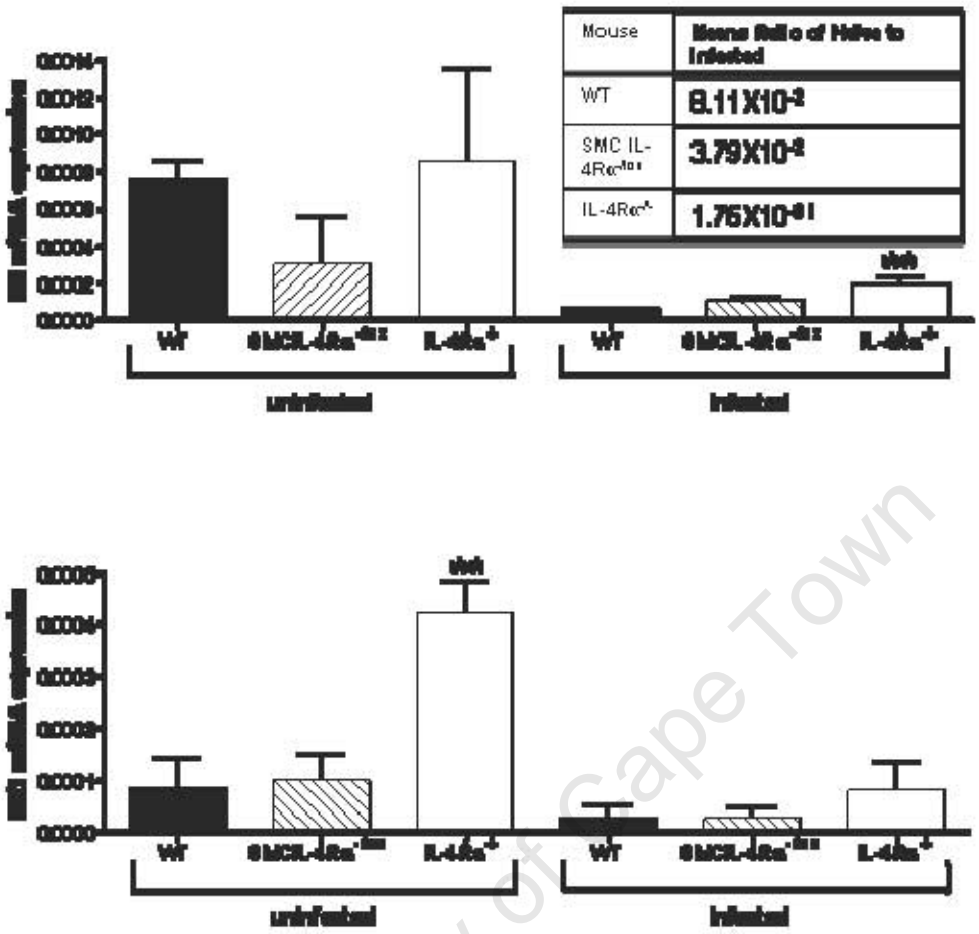


Figure 4
Intestinal expression of substance P and muscarinic receptors are suppressed during *S. mansoni* infection. mRNA was extracted from intestine of naive mice and *S. mansoni* infected mice 8 weeks p.i. Synthesized cDNA was probed with primers to (A) M3 or (B) NK1. Expression levels were normalised against 12S ribosomal mRNA. M3 data was pooled from two experiments. n≥3 mice per group. **P<0.01, NK1 data is preliminary results from one experiment.

2.3 DISCUSSION

In this study, we examined the role of IL-4Rα$^{-/-}$ on smooth muscle cells by utilizing a recently characterised cell specific gene deficient mice, SM-MHC^{cre} IL-4Rα$^{-/lox}$, disrupted for the expression of IL-4Rα on smooth muscle cells specifically (Horsnell *et al.*, 2007). The results obtained demonstrate that IL-4/IL-13 responsiveness by SMC is involved in efficient egg excretion during acute schistosomiasis. We further demonstrated by more rapid mortality and increased weight loss that SM-MHC^{cre} IL-4Rα$^{-/lox}$ mice are more susceptible to *S. mansoni* compared to control WT mice. Surprisingly, this increased susceptibility can not be explained by an increase of intestinal inflammation nor by a dysregulation of the immune response to egg antigens. Moreover, though muscarinic receptor M3 level of expression in the intestine of infected mice is reduced compared to naïve mice, M3 mRNA is similarly transcribed in SM-MHC^{cre} IL-4Rα$^{-/lox}$ or WT mice. In contrast, the M3 receptor expression was significantly higher in IL-4Rα$^{-/-}$ infected mice.

Shortening of villi, goblet cell hyperplasia and localized proliferation of SMC along with granuloma formation are the main pathological features in the intestine observed during schistosomiasis. Granulomatous lesions are accompanied with an increased gastrointestinal motility resulting to SMC hypercontractility (Moreels *et al.*, 2001; Moreels *et al.*, 2004). These changes have been associated with the T_H2 response induced by egg antigens. The latter is essential for survival during the acute schistosomiasis(Pearce *et al.*, 2004; Pearce & MacDonald, 2002). This suggests that the T_H2 response has a direct or indirect effect on both the hematopeotic and the non-hematopoetic cell types. While direct effects of the major T_H2 cytokines IL-4 and IL-13 have been shown during *S.mansoni* infection on hematopoetic cells, no direct specific effect of the cytokines on the function or role of IL-4/IL-13 responsiveness on non-hematopoetic cells have been shown during *S.mansoni* infection.

As mentioned above *S.mansoni* affects SMC proliferation and hypercontractility and (in other infection models) this has been shown to be Stat 6 dependant

(Akiho *et al.*, 2002). With the availability of the SMC specific IL-4Rα deficient mice (characterised by Horsnell et.al. 2007) a unique opportunity was presented to determine if there is an important role for IL-4/IL-13 responsiveness on SMC's during *S.mansoni* infection. Therefore we infected these mice and compared them to wild type mice and the global IL-4Rα deficient mice.

As previously published and supported in our results, mice disrupted for IL-4Rα globally and infected with *S. mansoni* do not survive the acute phase (Herbert *et al.*, 2004). Their high susceptibility can be explained by an increased inflammation in the intestine along with a high T_H1 response and a retention of eggs in the tissue (Herbert *et al.*, 2004) and Fig. 1 and 2). In these mice, the uncontrolled T_H1-biased inflammation is thought to be responsible for the mucosal damage and consequently the entry of high amounts of bacterial endotoxins in the blood system ((Herbert *et al.*, 2004) and Fig. 2D). In the SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice however despite their increased susceptibility and their high egg burden, no increase in the magnitude of the gut inflammation was observed. Similarly, these mice were able to respond to egg antigens with a controlled T_H2 response and no increase of serum endotoxin was observed. From these results, we conclude that IL-4/IL-13 responsive SMC play a major role at the peak of egg production which coincides with the peak of the T_H2 response.

SMC contraction is induced by autonomic nervous system and/or mechanically in response to stretch (Ishii & Kurachi, 2006; Matsui *et al.*, 2002). Upon signalling through the parasympathetic nervous system acetylcholine is released from neurons and mediates SMC contraction by binding to muscarinic receptors (Goyal & Hirano, 1996). M3 is considered the dominant muscarinic receptor in the intestine(Ehlert *et al.*, 1999). However other receptors are able to induce contraction in intestinal SMC (Holzer & Holzer-Petsche, 1997). Among these, Nk1 responds to the tachykinin namely substance P and is involved. During other helminth infection M3 receptor expression was shown to be upregulated in

Nippostrongylus brasiliensis infection WT mice in contrast to infected IL-4Rα$^{-/-}$ or SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice (Horsnell *et al.*, 2007). Consequently, a decreased gastrointestinal contractility may have lead to the observed disruption in worm expulsion (Horsnell *et al.*, 2007).

In contrast to *N. brasiliensis* infection, there is no evidence to date that suggests that hypercontractility is necessary for egg expulsion. However, we observed an increased susceptibility to infection associated with increased egg burden in mice disrupted for IL-4Rα in SMC. This result strongly suggests that IL-4/IL-13 responsive SMC play a role in egg migration and excretion. M3 and NK1 receptors are usually upregulated during hypercontractility (Ehlert *et al.*, 1999; Matsui *et al.*, 2002), however the downregulation of transcription of M3 and NK1 may suggest that these neurotransmitters are not involved during acute schistosomiasis. However it is necessary to confirm this mRNA result by conducting protein analysis using immunohistochemical staining or western blotting to determine if the protein expression reflects the mRNA levels. It could be that these receptors are stored and that signalling through IL-4Rα on SMC cells results in release of these receptors and not necessarly mRNA expression. Even this however would still be indirect evidence and further studies must be conducted in order to test whether the hypercontractility induced by the intestinal myenteric plexus is altered through disruption of IL-4Rα on SMC during schistosomiasis. Moreels et al. measured contractility of isolated muscle strips from the intestine and showed that *S.mansoni* infected mice had increased contractility from 8 weeks post infection compared to non-infected tissue (Moreels *et al.*, 2001). In addition they showed that this contractility was most significantly increased at 12 weeks post infection. They suggested that although the proliferation of smooth muscle was significantly elevated during the in acute phase that contractility was much more affected during the chronic phase (Bogers *et al.*, 2000; Moreels *et al.*, 2001; Moreels *et al.*, 2004). Therefore the M3 receptor expression may only increases in the chronic phase and that our future studies showed include chronic phase analysis. A short coming in these studies

by Moreels et al. is that they do not show or suggest the effect of contractility on egg mobility or expulsion (Bogers *et al.*, 2000; Moreels *et al.*, 2001). If hypercontractililty occurs in WT mice in the chronic phase or hypocontractility, in IL-4Rα deficient mice, it does not explain the decrease in egg expulsion during the acute phase in IL-4Rα deficient. Therefore contractility studies should be conducted at both time points to determine when contractility is affected to assess whether changes in contractility can be associated with egg expulsion.

Since SMC's do not only form part of the intestine but also blood vessels/arteries, the respiratory system and eyes, therefore changes in smooth muscle function (contractility) in other tissues may also explain the earlier mortality. Portal hypertension has been linked to mortality in humans and in mice studies (Kopke-Aguiar *et al.*, 2002; Mentink-Kane *et al.*, 2004; Njenga *et al.*, 1998). Increased egg burden in deficient mice, fibrosis and poor muscle function may increase the blood pressure in the portal system and result in earlier mortality (Njenga *et al.*, 1998; Vennervald *et al.*, 2004). Further studies may prove this by measuring blood pressure changes directly in the portal system or measuring portal hypertension markers such as hyaluronic acid (Kopke-Aguiar *et al.*, 2002).

In conclusion, we suggest that during schistosomiasis IL-4/IL-13 responsive SMC play a role in improving parasite egg excretion and therefore reduced intestinal morbidity and mortality as a consequence hastened portal hypertension.

CHAPTER 3

CHAPTER 3

IL-4/IL-13 independent goblet cell hyperplasia in experimental helminth infections.

3.1 INTRODUCTION

Interleukin (IL)-4 and IL-13 are related cytokines and the dominant mediators of TH2 immune responses (McKenzie *et al.*, 1998; McKenzie *et al.*, 1999; Mohrs *et al.*, 1999). Signalling by both cytokines is dependent on binding to heterodimeric receptors containing the IL-4 receptor α chain (IL-4Rα. Ligand binding results in intracellular signalling pathways activating the TH2 defining transcription factors STAT-6 and/or GATA-3 (Andrews *et al.*, 2002; Metwali *et al.*, 2002). This polarisation to a T_H2 immune response is essential for the successful resolution of a number of helminth infections (Barner *et al.*, 1998; Dehlawi *et al.*, 2006; Herbert *et al.*, 2004; Horsnell *et al.*, 2007; Michels *et al.*, 2006).

Actual worm expulsion, in nematode infections, is associated with increased IL-13/ IL-4Rα/ STAT-6 dependent intestinal smooth muscle contractions, epithelial cell turnover and goblet cell hyperplasia (Akiho *et al.*, 2002; Cliffe *et al.*, 2005; Zhao *et al.*, 2003). Infections of IL-$4^{-/-}$, IL-$13^{-/-}$, IL-$4R\alpha^{-/-}$ and Stat $6^{-/-}$ mice with the nematodes *Trichuris muris, Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* have demonstrated a positive relationship between polarisation to a TH2 immune response, goblet cell hyperplasia and worm expulsion (Else *et al.*, 1994; Finkelman *et al.*, 2000; Finkelman *et al.*, 1997; Schopf *et al.*, 2002; Shea-Donohue *et al.*, 2001; Webb *et al.*, 2007). In support of a role for goblet cell derived mucus in worm expulsion *in vitro* experiments have demonstrated increased viscosity of mileu surrounding *N. brasiliensis* at an equivalent density to intestinal mucus inhibits worm movement (Lee & Biggs, 1990). Moreover, isolation of the goblet cell secreted protein RELMβ/FIZZ2 and incubation with parasitic nematodes *in vitro* results in impaired chemotactic function in the worm

(Artis *et al.*, 2004). These observations have led to TH2 induced goblet cell hyperplasia being considered a key mechanistic factor in resolving gastrointestinal related nematode infections (Allen *et al.*, 1988; Miller, 1987; Onah & Nawa, 2000)

Intestinal goblet cell hyperplasia in *Schistosoma mansoni* (*S. mansoni*) infections is driven by parasite eggs traversing the intestine (Fallon *et al.*, 2000a; Herbert *et al.*, 2004), as opposed to nematode infections where adult worms residing in the intestine induce the goblet cell responses (Herbert *et al.*, 2004; Horsnell *et al.*, 2007; McKenzie *et al.*, 1998). *S. mansoni* eggs produced by adults residing in the mesenteric venules move from the blood vessels through the intestine passing to the lumen. This movement of eggs generates considerable tissue damage as well as inducing a strong mucosal response in the intestine (Herbert *et al.*, 2004; Weinstock & Boros, 1981). As with nematode infections, *S. mansoni* induced mucus production has been considered to be TH2 dependant (Ishikawa *et al.*, 1993; Ishikawa *et al.*, 1994; Onah & Nawa, 2000; Yamauchi *et al.*, 2006).

In this study we examined goblet cell hyperplasia in response to infection with the nematodes *N. brasiliensis* and *Syphacia obvelata* and the trematode *S. mansoni*. As already published *N. brasiliensis* infection induced a goblet cell hyperplasic response dependent on IL-4/IL-13/IL-4Rα expression (Horsnell *et al.*, 2007). However, infection with the nematode *S. obvelata* did not increase goblet cell hyperplasia in the host colon, irrespective of IL-4Rα expression. Such data demonstrates that IL-4Rα driven goblet cell hyperplasia may not be essential for the clearance of all gastro-intestinal nematode infections. Furthermore, we also show *S. mansoni* induced goblet cell hyperplasia to be independent of IL-4/IL-13 responsiveness. This data represents the first demonstration of goblet cell hyperplasia and mucus production in response to helminth infections being independent of IL-4/IL-13.

3.2 RESULT

3.2.1 *N. brasiliensis*[1] infection induces IL-4/IL-13 dependent goblet cell hyperplasia while *S. obvelata*[2] infection does not induce goblet cell hyperplasia.

Examination of IL-4/IL-13 dependent goblet cell hyperplasic responses in the intestinal niches utilised by the nematodes *N. brasiliensis* and *S. obvelata* infections was carried out in WT, IL-4/IL-13$^{-/-}$, IL-4$^{-/-}$ and IL-4Rα$^{-/-}$ mice.

N. brasiliensis infected WT mice demonstrated significantly higher levels of goblet cell hyperplasia in the small intestine at both days 7 and 10 post infection (PI) when compared to naïve mice (Figure 1A). However, no significant increase in the number of goblet cells in the intestine could be detected in infected and naïve IL-4/IL-13$^{-/-}$ or IL4Rα$^{-/-}$ mice when compared to naïve controls (Figure 1A). Examination of intestinal worm burdens in WT mice showed resolution of infection by day 10 PI. Both IL-4/IL-13$^{-/-}$ and IL-4Rα$^{-/-}$ mice failed to expel adult worms by day 10 PI (Figure 1B). These data confirm *N. brasiliensis* clearance to be associated with an IL-4/13/IL-4Rα dependent goblet cell hyperplasia.

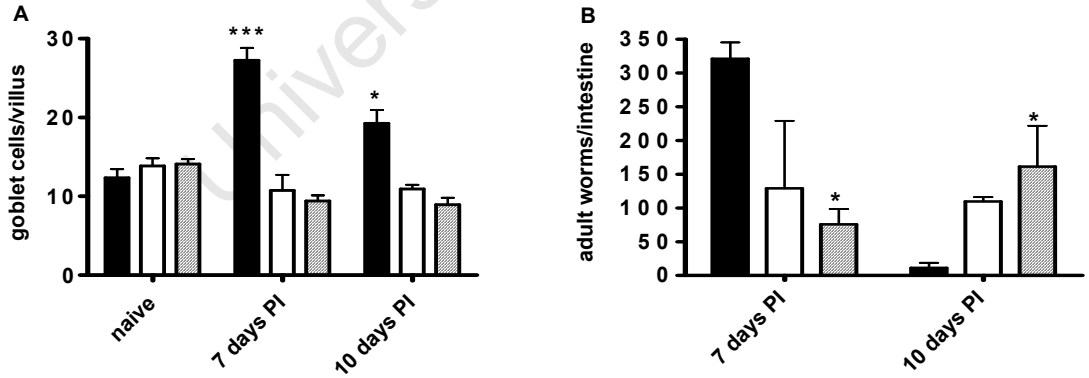


Figure1: Goblet cell hyperplasia in *N. brasiliensis* infection.
A) Goblet cell quantification naïve mice and *N. brasiliensis* infected WT (solid bar), IL-4Rα$^{-/-}$ (open bar) and IL-4/IL-13$^{-/-}$ (gray bar) mice 7 days and 10 days PI in small intestine sections.
B) Intestinal adult worm burden 7 and 10 days post infection.
Data representative of two experiments are shown. Data are means of four mice per group ± SEM. * $P < 0.05$; *** $P < 0.001$ (significantly different from naïve mice).

[1] ***N. brasiliensis* infection studies performed by W. Horsnell, L.Smith and R.Marillier**
[2] ***S. obverlata* infection studies performed by C. Michels**

S. obvelata adult worm burdens in infected wild type mice are only detectable by day 28 PI (approx. 1.25 worms/ caecum), this burden peaks by day 35 PI to approximately 21 worms/ caecum. Infection of IL-4Rα$^{-/-}$ mice results in considerably higher worm burdens (approx. 251 worms/ caecum at day 28 PI and 400 worms/caecum at day 35 PI) when compared to wild type mice (Michels *et al.*, 2006). In contrast to *N. brasiliensis* infected mice, no induction of goblet cell hyperplasia in the colon of *S. obvelata* infected WT, IL-4Rα$^{-/-}$ and IL-4$^{-/-}$ mice was seen (Figure 2A and B).

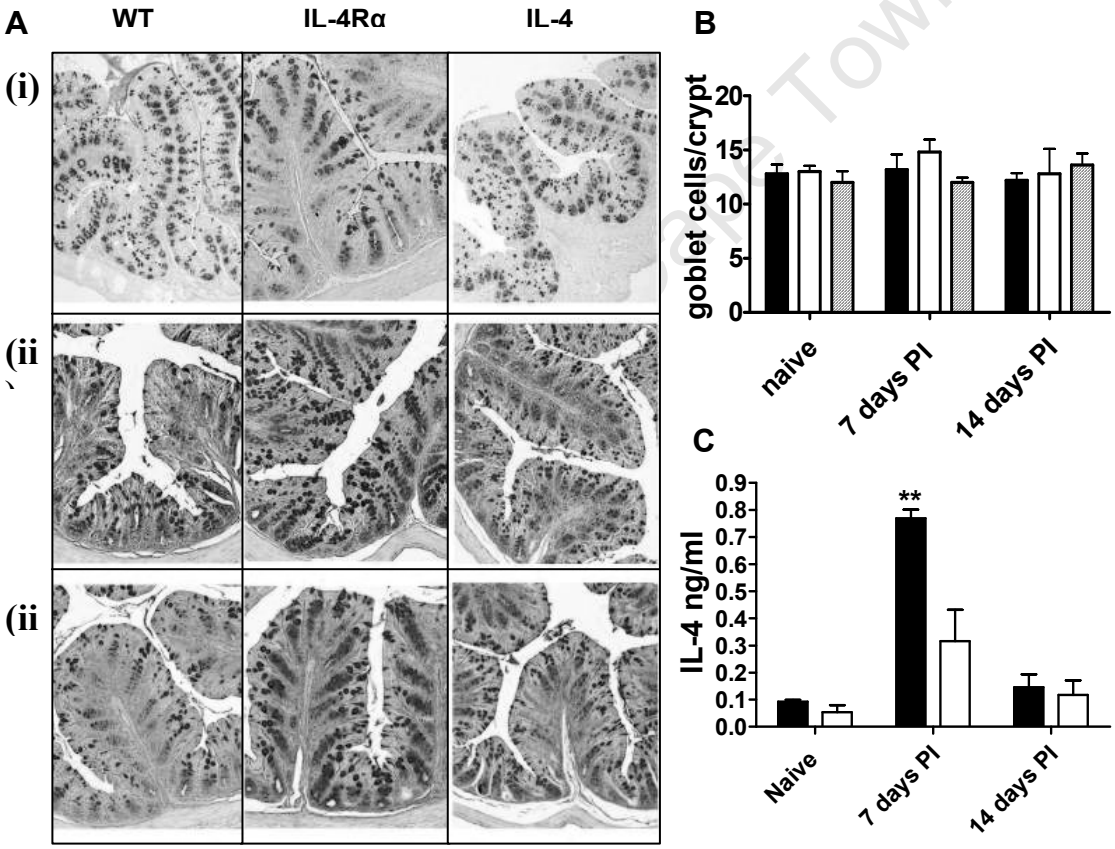


Figure 2: Goblet cell hyperplasia in *S.obvelata* infection.
(A) Photomicrograph of colon from WT, IL-4Rα$^{-/-}$ and IL-4$^{-/-}$. Representative pictures of colon sections are shown from both naïve (i) and pinworm infected mice at 7 (ii) and 14 days PI (iii). Sections were stained with PAS to identify goblet cells. 100x magnification.
(B) Quantification of goblet cells per crypt in colon 7 and 14 days PI in WT (solid bar), IL-4Rα$^{-/-}$ (open bar) and IL-4/IL-13$^{-/-}$ (gray bar). Data representative of two experiments are shown. Data are means of four mice per group ± SEM.
(C) *S. obvelata* dependent IL-4 secretion from anti-CD3 restimulated splenocytes. WT (solid bar), IL-4Rα$^{-/-}$ (open bar). **, P< 0.01 (significantly different from naive mice). Data representative of two experiments showing means for four mice/group ± SD.

This lack of a goblet cell response was irrespective of heightened levels of the TH2 cytokine IL-4 at day 7 PI ($p<0.01$) in WT restimulated splenocytes isolated from infected mice (Figure 2C) WT IL-4 levels declined to that found in naïve mice at day 14 PI. IL-4Rα$^{-/-}$ mice failed to demonstrate any significant increase in IL-4 production when compared to naïve mice.

Together these data demonstrate that *S. obvelata* infections do not induce a colonic mucus response even though levels of IL-4 and other TH2 cytokines are significantly increased (Michels *et al.*, 2006)

3.2.2 *Schistosoma mansoni* [3] induces goblet cell hyperplasia in the intestine in an IL-4/IL-13 independent manner.

S. mansoni infection induces a strong TH2 immune response and goblet cell hyperplasia related to parasite egg production (Fallon *et al.*, 2000a; Grzych *et al.*, 1991) In order to confirm the role of parasite eggs in induction of goblet cell hyperplasia, we analysed the hyperplasic response at 5 weeks PI (before the peak of egg production) and at the peak of parasite egg production; 8 weeks PI. While no difference in the number of goblet cells could be detected in the intestine of naïve or infected WT at 5 weeks PI (data not shown), a strong induction of goblet cell hyperplasia was detected at 8 weeks PI (Figure 3Aand 3B). To establish whether this hyperplasic response was dependent on IL-4/IL-13/IL-4Rα responsiveness we examined the intestines of infected IL-4/IL-13$^{-/-}$ and IL-4Rα$^{-/-}$ mice at 8 weeks PI. Here we found no difference in the numbers of eggs accumulating in the small intestine between WT, IL-4/IL-13$^{-/-}$ and IL-4Rα$^{-/-}$ mice (Figure 3A). Goblet cell hyperplasia in the small intestine of all infected mouse groups was significantly elevated above naïve controls (Figures 3A). Furthermore, equivalent levels of goblet cell hyperplasia were found in the intestine of all infected mice groups (Figures 3A). To demonstrate if IL-4/ IL-13/ IL-4Rα independent goblet cell hyperplasia occurred throughout the intestine we

[3] *S.mansoni* infection studies performed by R. Marillier

also examined the colon of both naïve and infected mice. As with the small intestine goblet cell hyperplasia was elevated above naïve controls in all mouse groups and no differences were found between infected groups (Figure 4). Together these results demonstrate IL-4/IL-13/IL-4Rα independent goblet cell hyperplasia in the intestine of mice infected with *S. mansoni*.

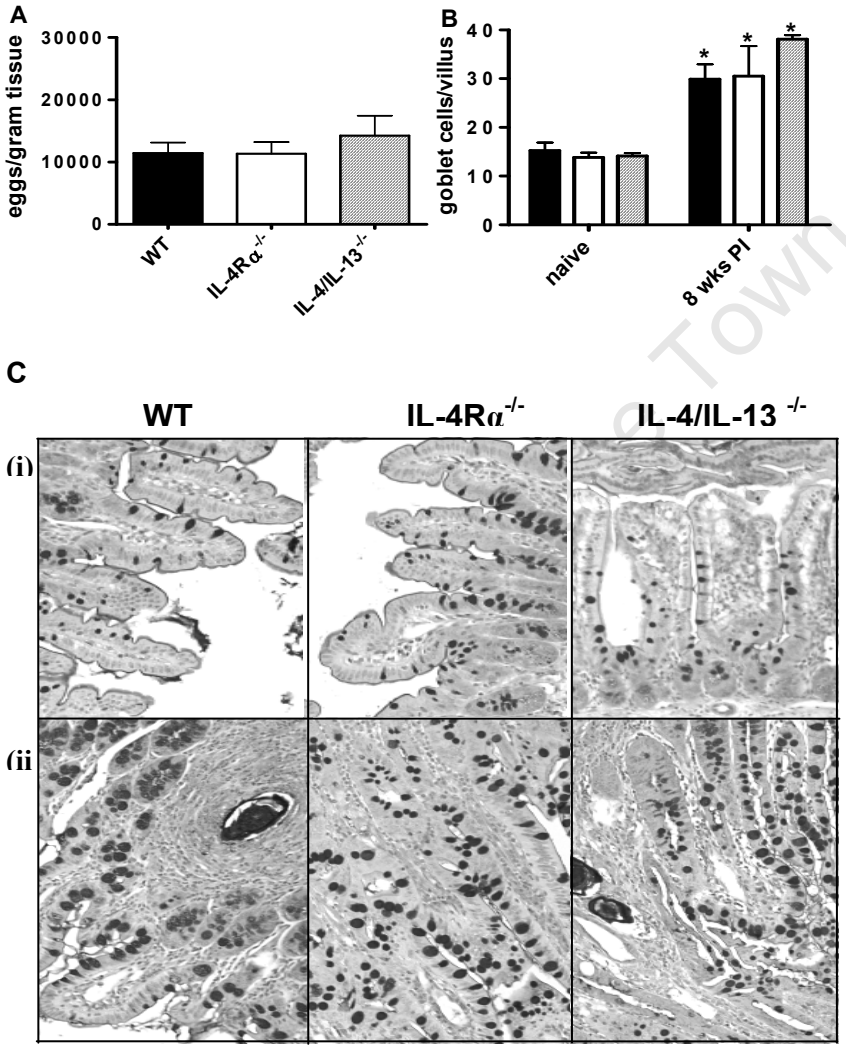


Figure 3: Goblet cell hyperplasia in ileum during *S.mansoni* infection.

(A) *S.mansoni* egg content in the ileum of WT (solid bar), IL-4Rα$^{-/-}$ (open bar) and IL-4/IL-13$^{-/-}$ (gray bar) mice at 8 weeks PI. Data are pooled from 2 to 4 individual experiments. Data are means of these experiments ± SEM. (B) Quantification of goblet cells per villus in ileum of naïve mice and 8 weeks PI from WT (black bars), IL-4Rα$^{-/-}$ (open bar) and IL-4/IL-13$^{-/-}$ (gray bar). Data representative of three experiments are shown. * P< 0.05 (significantly different from naïve mice). (C) Photomicrograph of ileum from WT, IL-4Rα$^{-/-}$ and IL-4/IL-13$^{-/-}$. Representative pictures of ileum sections are shown from both naïve (i) and *S. mansoni* infected mice at 8 weeks PI (ii). Sections were stained with PAS to identify goblet cells. 100x magnification.

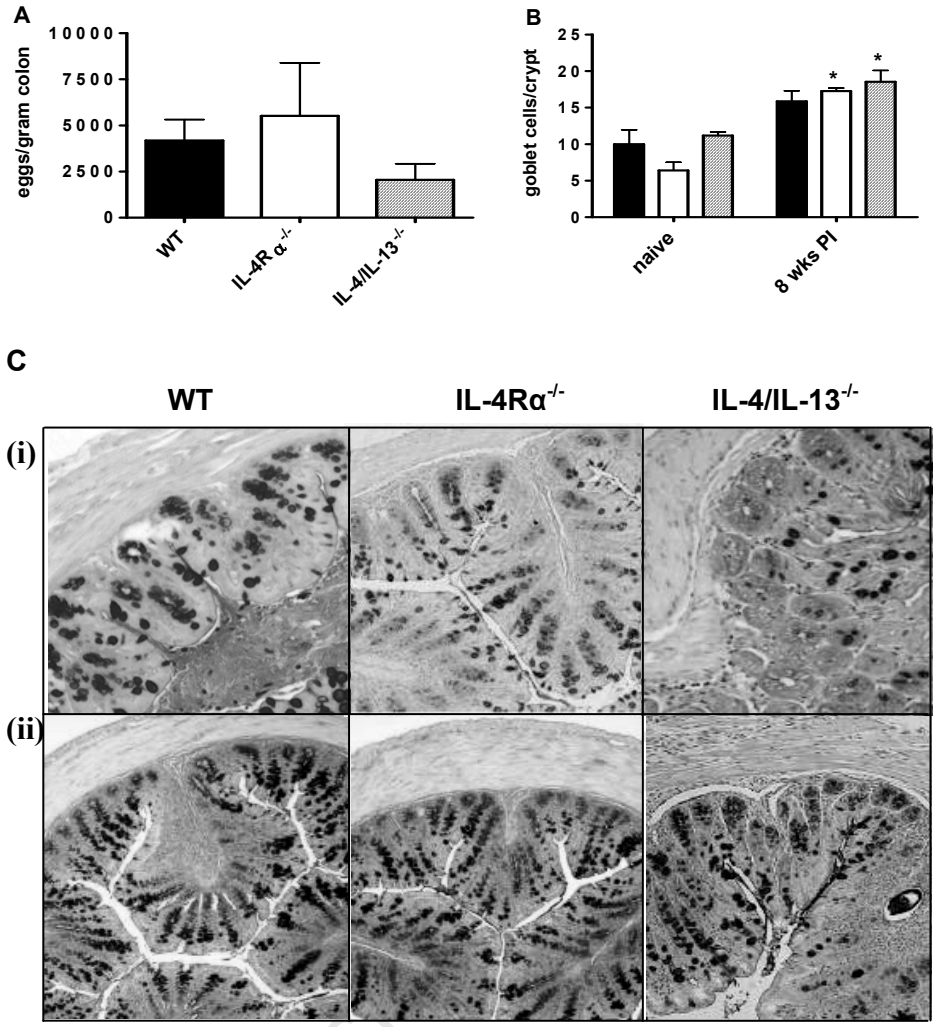


Figure 4: Goblet cell hyperplasia in the colon during *S.mansoni* infection.

(A) Tissue egg content of the colon from *S. mansoni* infected WT (solid bar), IL-4Rα$^{-/-}$ (open bar) and IL-4/IL-13$^{-/-}$ (gray bar) mice at 8 weeks PI. Data are pooled from 2 to 4 individual experiments. Data are means of these experiments ± SEM.

(B) Quantification of goblet cell number per villus in colon of naïve mice and 8 weeks PI from WT (solid bar), IL-4Rα$^{-/-}$ (open bar) and IL-4/IL-13$^{-/-}$ (gray bar) mice. Data representative of three experiments are shown. * $P < 0.05$ (significantly different from naïve mice).

(C) Photomicrograph of colon from WT, IL-4Rα$^{-/-}$ and IL-4/IL-13$^{-/-}$. Representative pictures of colon sections are shown from both naïve (i) and *S. mansoni* infected mice at 8 weeks PI (ii). Sections were stained with PAS to identify goblet cells. 100x magnification

3.3 DISCUSSION

Our data demonstrates that (i) goblet cell hyperplasia is dependent on helminth species and (ii) IL-4/IL-13 responsiveness is not required for induction of *S. mansoni* egg induced goblet cell hyperplasia.

It has previously been demonstrated that *N. brasiliensis* (Horsnell *et al.*, 2007) and *S. obvelata* (Michels *et al.*, 2006) infected IL-4$^{-/-}$, IL-13$^{-/-}$ and IL-4Rα$^{-/-}$ mice have impaired worm expulsion, while in *S. mansoni* infections IL-4/IL-13 signalling is essential for host survival (Herbert *et al.*, 2004). A common feature of both *N. brasiliensis* and *S. mansoni* infections is the hosts' goblet cell hyperplasic response to the parasite. Such responses have previously been considered to be dependent, in part at least, on the hosts TH2 polarised immune response (Horsnell *et al.*, 2007; Leeto *et al.*, 2006). From the data presented here and in other studies this does indeed appear to be the case in *N. brasiliensis* infections(Barner *et al.*, 1998; Herbert *et al.*, 2004; Horsnell *et al.*, 2007). Work on other parasitic nematode models such a *T. muris* also show a TH2 dependent worm expulsion and goblet cell response (Schopf *et al.*, 2002). However, in this study we have demonstrated that this may not be the case for all intestinal nematode infections.

Following oral infection with *S. obvelata* eggs, larvae emerge in the hosts small intestine at 7 day PI (Stahl, 1963). From here the larvae migrate, mature and establish the definitive infection in the hosts cecum and colon. We found the hosts TH2 immune response to peak at day 7 PI and then decreases from at least day 14 PI. Previous work has shown that by day 35 PI this response is undetectable (Michels *et al.*, 2006). Together, these data demonstrate a transient TH2 response to this infection. TH2 responses in other intestinal nematode infections result in strong goblet cell hyperplasic responses (Else *et al.*, 1994; Shea-Donohue *et al.*, 2001; Urban *et al.*, 2000; Webb *et al.*, 2007). However, mice infected with *S. obvelata* failed to generate hyperplasic goblet cell responses, suggesting that TH2 induction of intestinal mucus responses is not a

common feature of intestinal nematode infections, or that the TH2 response needs to be sustained. Other factors such as prostaglandins (Madden *et al.*, 2002), cholinergic (Zhao *et al.*, 2003) and non-cholinergic (Zhao *et al.*, 2003) agonist may also play a role. Additionally, the different niches occupied by various species of parasitic nematodes could effect the host response to them (Grencis, 2003). *S. obvelata* infections do not cause major pathology in the intestine (Michels *et al.*, 2006) as opposed to *N. brasiliensis* and *T. muris* which cause considerable histological damage to the hosts intestinal architecture (Horsnell *et al.*, 2007; Onah & Nawa, 2000). Such differences in worm pathogenicity may explain the lack of a goblet cell response in *S. obvelata* infections, irrespective of the hosts TH2 polarisation (Michels *et al.*, 2006).

S. mansoni infection induces a strong TH2 response initiated by worm egg production at week 4 PI and persists throughout the infection (Pearce & MacDonald, 2002). Associated with this are significant levels of goblet cell hyperplasia in the intestine (Fallon *et al.*, 2000a; Grzych *et al.*, 1991). *S. mansoni* egg antigens have previously been shown to also induce goblet cell hyperplasia in the lung in a IL-4Rα dependent manner (Leeto *et al.*, 2006). However the role of IL-4Rα in goblet cell hyperplasia in the intestine during the live infection has not been shown. An explanation for the IL-4Rα independent hyperplasia described here could be the mode of *S.mansoni* infection and its interaction with the hosts' tissue. *S. mansoni* eggs cause pathology from the adventitial surface of the intestine, as opposed to nematodes driving the pathology from the lumen. We propose that the severe tissue damage resulting from the eggs migration from the adventitial surface to the lumen is capable of initiating a goblet cell response, independently of IL-4 and IL-13 signalling during *S.mansoni* infection.

In addition to IL-4/IL-13 other cytokines may act to induce goblet cells hyperplasia. IL-9 and IL-5 have previously been shown to play a role in directly inducing IL-4/IL-13 independent goblet cell hyperplasia in lung models (Longphre *et al.*, 1999; Nath *et al.*, 2007). IL-9 overexpressing transgenic mice infected with

S.mansoni do have increased goblet cell hyperplasia (Fallon *et al.*, 2000b). However IL-9 transgenic mice also had increased IL-4 and IL-13 compared to wild type mice, and therefore it cannot be concluded that IL-9 directly increases goblet cell hyperplasia. Furthermore IL-9 levels are decreased in *N. brasiliensis* infected IL-4Rα$^{-/-}$ mice (Horsnell *et al.*, 2007). As such IL-4/IL-13 independent intestinal goblet cell hyperplasia may not be due to increased IL-9. No clear reports linking IL-5 to goblet cell hyperplasia during *S.mansoni* infection have reported. As IL-4Rα$^{-/-}$ mice have decreased IL-5 expression it is also unlikely that IL-5 induces intestinal goblet cell hyperplasia in *S.mansoni* infections (Herbert *et al.*, 2004).

In conclusion our results demonstrate for the first time that intestinal goblet cell hyperplasia in response to parasitic helminth infections can occur independently of IL-4/IL-13 signalling and that intestinal nematode infections may not always induce a goblet cell response.

GENERAL DISCUSSION

GENERAL DISCUSSION

In the murine model for *Schistosoma mansoni* infection the host survives by inducing a strong T_H2 cytokine (namely IL-4 and IL-13) driven response. Acute phase protection has therefore been associated with IL-4Rα dependant granuloma formation and expulsion of eggs.The wide range of pathophysiological features during *S.mansoni* infection indicates that IL-4/IL-13 responsiveness is necessary on a number of cells to mediate protection during infection (Flores Villanueva *et al.*, 1994). IL-4Rα is found on both haematopoetic and non-haematopoetic cells. However the exact role of IL-4/IL13 responsiveness on these cells is not known.

S.mansoni infection induces a number of changes to the host tissue including granuloma formation, goblet cell hyperplasia and smooth muscle hyperplasia and hypercontractility and fibrosis. Much controversy still exists over the function of these changes during schistosomiasis. It is not clear whether the TH2 response is induced by the host to protect itsself or if the helminth induces this type of response to ensure its long term survival in the host (Maizels & Yazdanbakhsh, 2003)).

Upon egg deposition in tissues, granuloma develop around the eggs. Granuloma formation may have a dual function during schistosomiasis . One line of evidence suggests that granuloma formation aids in the expulsion of eggs. In support of this it has been shown that when granuloma do not form in IL-4Rα deficient mice egg expulsion is abrogated (Herbert *et al.*, 2004). Secondly, evidence suggests that granuloma formation is necessary for suppression of inflammation due to the fact that it encapculates the egg which may protect of the host from harmful pathogens. Furthermore, we show here that cells, and in particular IL-4/IL-13 responsive cells, have a specific location around the egg within the granuloma. As previously discussed in chapter one, the functional markers YM-1 and MMR expressed in the granuloma are both implicated in immune suppression which supports the former argument that granuloma suppress inflammation. Conversely

however, fibrotic granuloma formation in the liver and in the intestine may obstruct tissue function and lead to hypertension and poor gastro-intestinal function in humans and mice (Farah *et al.*, 2000; Geboes *et al.*, 1990; Gryseels, 1992; Pearce & MacDonald, 2002). Granuloma formation is therefore both necessary for egg expulsion and for suppression of inflammation, but may lead to pathology in the chronic phase.

In addition to granuloma formation, goblet cell hyperplasia and smooth muscle cell hypercontractility which also occur during schistosomiasis are common changes that occur in most helminth infections that are associated with the induction of the T_H2 immune response such as *N.brasiliensis*, *T.muris* and *H. polygrus*. During infection with luminal dwelling nematodes such goblet cell hyperplasia and smooth muscle cell hypercontractility increase gastrointestinal transit which aids worm expulsion(Grencis, 1997; Horsnell *et al.*, 2007). During *Schistosoma mansoni* infection such changes do not appear to be relevant, because adult worms do not reside in lumen of the intestine where the changes could have an effect (Wilson *et al.*, 2007).

It has been the aim of our lab and others to determine the relevance of the T_H2 induced factors in protection of the host during helminth infections. The novel finding here is that the correct localisation of granuloma cells and IL-4/IL-13 responsive smooth muscle cells is required for egg expulsion which is associated with decreased susceptibility. Therefore to tjhis suggests that T_H2 to dependent events appear to aid egg expulsion. On the other hand, the TH2 response is necessary for immune regulation (Balic *et al.*, 2006; Grencis, 1997; Schopf *et al.*, 2002). In SM-MHC^{cre}IL-4Rα$^{-/lox}$,mice IL-4/IL-13 responsive macrophages are still present (MMR positive macrophages, unpublished data). This may explain why granuloma formation is similar to WT mice in the acute phase despite higher egg burdens SMC mice.

An unexpected finding was that goblet cell hyperplasia was induced by *S.mansoni* was independent of IL-4/IL-13 responsiveness. However we were undable to determine whether there was as significant function for the induction of goblet cell hyperplasia or if it was a consequence of intestinal damage.

Whereas *S.mansoni* has been considered a huge burden on the developing world, the lack of such infection has been associated with an increase in auto-immune disease and allergy (such as colitis and Crohns disease as well as symptoms of allergy such as asthma), in the developed/first world countries.(Yazdanbakhsh *et al.*, 2002). The hygiene hypothesis suggests that due to lower exposure to infectious agents and parasites, by the exploitation of antibacterial and antihelminthic agents, individuals develop hypersensitive responses to allergens and/or autoimmune disease. Moreels *et al*. (2004) showed that co-infection with *S.mansoni* improved grastrointestinal transit in TNBS induced colitis (T_H1 associated disease) (Moreels *et al.*, 2004). Schistosomiasis involves similar pathological processes to allergy (such as mucus production and hyper-responsiveness) but when individuals with schistosomiasis are exposed to allergens they showed decreased severity of allergic responses (van den Biggelaar *et al.*, 2000). This regulation of the allergic response was suggested to be a result of the induction of IL-10 and the induction of the T_H2 response (namely IgE). . In accordance with these findings IL-10 is upregulated during *S.mansoni* infections (Herbert *et al.*, 2004; Hoffmann *et al.*, 2000). IL-10 together with TGFβ have strong suppressive functions. They dampen down both T_H1 and T_H2 immune responses and therefore it is the induction of these pathways that are thought to be important the suppression of first world disorders (mentioned above)(Maizels & Yazdanbakhsh, 2003). During *S.mansoni* infection of $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ however, IL-10 was elevated in splenocytes, lymph nodes and in the tissue but mice were still more susceptible than wild type mice (Herbert *et al.*, 2004). This suggests that AAMϕ may act together with IL-10 to reduce inflammation during *S.mansoni* infection and that though IL-10 has a suppressive function in allergy it may still require the IL-4/IL-

13 activity. Thus if IL-10 or AAMϕ are to be used in anti-inflammatory therapy, effective strategies must still be determined since these factors do not act in isolation (Balic *et al.*, 2006; Yazdanbakhsh *et al.*, 2002).

In summary these results show that IL-4 and IL-13 mediate a number of immune functions by their pleiotropic nature but that *S.mansoni* may also be directly involved in inducing some of these functions. We provided further evidence that egg expulsion is mediated by the host and also that the formation of correct granuloma formation can mediate decreased susceptibility even when the egg burden is high. $YM1^{hi}MMR^{+}$ cells at the core of the granuloma appear to play an important role in this regard. This thesis further resolves our understanding of the role of IL-4Rα during *S.mansoni* induced immunopathology.

Future work and concluding remarks

This dissertation provides further insight into the role of IL-4/IL-13 signalling on haematopoetic and non-haematopoetic cells. Several aspects require further investigation:

A novel finding was the identification of a subpopulation of macrophages that express YM-1 independently of the IL-4Rα in vitro and the localisation of a highly YM-1 positive population of cells in the area closely surrounding the eggs in both liver and intestinal granulomas in a macrophage IL-4Rα dependent manner. While AAMΦ do express YM-1 and MMR these markers may also be found on other cells for example neutrophils and dendritic cells. Future work will incorporate the use of flow cytometry, confocal microscopy and microarrays to determine the expression profile of these YM-1 and MMR positive populations. Furthermore, to determine the activation state of these cells, additional markers should be included in this analysis. Additional markers of AAMϕ have been identified including arginase, Fizz1, Relmα, MS-1 high molecular weight protein

(MS-1-HMWP) and RM3/1 (Gordon, 2003; Gratchev *et al.*, 2001; Loke *et al.*, 2002). These markers however may be induced by other cells (neutrophils, epithelial cells, resident macrophages, dendritic cells) and also by IL-4Rα independent mechanisms; therefore analysis should be completed in combination with IL-4Rα staining and macrophage markers (Harbord *et al.*, 2002).

Following characterisation of the cells expressing YM-1 close to the egg in WT mice, the next question that needs to be answered is if YM-1 has a direct role during *S.mansoni* infection. Chang et al. reported the isolation and purification of YM-1 from peritoneal exudates and cell supernatants. (Chang *et al.*, 2001; Tsai *et al.*, 2004). Using a similar approach to purify YM-1, future experiment should test whether YM1 treatment has the ability to rescue IL-4Rα-/- mice from pathology. Alternatively, YM1 positive cells isolated from WT and $LysM^{cre}$IL-4$Rα^{-/lox}$ mice could be tested or compared for their ability to decrease susceptibility in $LysM^{cre}$IL-4$Rα^{-/lox}$ and IL-4$Rα^{-/-}$ mice. One concern however is the plasticity of macrophage in various inflammatory milieu (Edwards *et al.*, 2006; Gordon, 2003). A particular concern will be the re-differentiation of macrophages from alternative to classically activated macrophage phenotypes in IL-4$Rα^{-/-}$ mice where the T_H1 response is high. A number of cytokines such as IL-12 and IFNγ drive classical macrophage activation. Perhaps by using macrophages deficient in receptors or transcription factors which drive T_H1 responses, macrophages may be locked into an alternative phenotype and thereby avoiding a switch in the activation status upon injection into a new inflammatory environment. A third possibility to determine if YM-1 is necessary during *S.mansoni* infection, would be to generate YM-1 deficient mice (similarly to the $LysM^{cre}$IL-4$Rα^{-/lox}$ and IL-4$Rα^{-/l-}$ mice). Infecting these mice will reveal whether YM1 has a direct dominant role during *S.mansoni* infection..

YM-1 and MMR mediate immune suppression, but the mechanism is not known. Antigen presentation is an important process in the development of the immune

response. The results showed MHCII+ and B220+ cells appear at the periphery of granulomas suggesting that antigen processing may occur here. While studies have shown that DC's endocytose SEA (Perona-Wright *et al.*, 2006) future work should investigate the fate of SEA within the granuloma and the localization of antigen presenting cells. The ability to label parasites or their antigens and the use of two photon microscopy will allow the analysis of living tissue, the processing of antigen and trafficking of cells within the granuloma.

As shown in this thesis non-haematopoetic cell-specific expression of IL-4Rα also mediated protective responses during *S.mansoni* infection. IL-4 responsiveness on smooth muscle cells were found to be required for egg expulsion and decreased susceptibility. Recently our lab has acquired the equipment to measure contractility, which will allow us to determine if the failure to expel eggs efficiently is due to decreased contractility in IL-4Rα deficient mice. If this is proved to be the case further analysis will be required to determine is this is due to changes in factors produced by SMC such as receptor expression. While RNA analysis gives an indication of changes on the transcription level, protein assays (Western blots, immunostaining and binding assays) may be used to determine the changes in production of receptors responsible for contraction and also changes in affinity of the receptors for their ligands. It could also be argued however that changes in contractility which potentially lead to poor egg expultion are an indirect effect of damage induced by eggs and pro-inflammatory molecules (Pittschieler, 1999). Therefore analysis must be conducted to determine if there are increased damage and damaging factors such as NO in smooth muscle tissue and the myenteric plexus of the SM-SMC^{cre}IL-4Rα$^{-/flox}$ mice.

The optimisation and incorporation of these new technologies will facilitate our understanding of the regulation of inflammatory networks and will be useful in designing strategies to limit the degree of immunopathology induced by *S.mansoni* and other helminths *(Wilson et al., 2007)*.

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