The role of Interleukin-4 receptor alpha on smooth muscle cells during helminth infection

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Dedication

This thesis is dedicated to my dearly beloved mom Constance Selekaganyo Bogale and my heart's desire, Thato Brombacher—my son.

Acknowledgements

I would like to pass my gratitude and warm thank you to Prof. Brombacher for taking me on to join his amazing team of researchers. You provided this wonderful topic for my research and I have loved exploring the angles. Thank you for critically assessing my work and guiding me every step of the way. I am grateful to the ever so hardworking staff that made it possible for this work to flow. Thank you goes to the genotyping team that always cared to make sure I have enough mice for my experiments, and always went the extra mile to make sure I have the required mice on time. Thank you guys for being friends that offered shoulders for me to cry on. Thank you very much animal unit staff for taking care of my mice and always delivering them on time; even when I made mistakes on my forms with almost every order. Surgery department team—I don't know where to start. You worked with me, you brainstormed with me, you held my hand when it was tough, above all, you believed in me and kept rolling with the punches. I will never forget the number of times we attempted to locate those neurons...Thank you, thank you. I am thankful to Dr. Kellaway for having been there at the beginning of my career to establish ground for the purchase of the contractions measuring equipment and teaching me how to use the powerlab™. This was the foundation of my PhD, I will be forever thankful to you. Dr. Rob Smith my sincere thanks go out to you for teaching me all I know about the powerlab™ and organ bath. You took time off your busy schedule to come and train me, took my panicked phone calls, responded to my frantic emails, and patiently guided me through the storms of calibrating the equipment, understanding the readings, and even how to make buffer the right way. God bless you. Thank you Dr. Marillier for helping me acknowledge that "we are all in this together..." I'm thankful that you taught me histological techniques and gave me protocols to all immunological techniques I needed to learn. I extend my gratitude to Dr. Schwegmann for being an angel and holding my hand through all major aspects of my projects and for re-assuring me that "it will all work out" whenever I reached the end of the rope. Thank you Dr. Leeto for helping me from day 1 with contractions experiments that I couldn't handle on my own. I'm glad you were there to help with experiments when I was pregnant and when my baby was sick. I will never forget the day our "home-made" contractions measuring equipment broke and water spilled all over the laptops—we thought that was the end, but hey, look at us now. Thank you to Dr. Horsnell for being my co-supervisor. Berry, Gloria and D, you are amazing people—you wiped my tears and shared my joys. Saskia, I'm glad you showed up when you did. To Dr. Guler, thank you for caring. Special thanks go to the quiet room..."stick to the status quo". My final thank yous go to my loving, caring, and patient husband. Franky, I would marry you again. Thank you for your tolerance and support...

TABLE OF CONTENTS

LIST OF FIGURES

Part 1: General Introduction

Part 2: Optimization of Isolated Intestine Tissue Contraction Method

Part 3: *N. brasiliensis* infected IL-4Rα-deficient mice tissue contraction

LIST OF TABLES

Part 3: *N. brasiliensis* infected IL-4Rα-deficient mice tissue contraction

LIST OF PICTURES

Part 4: Discussion

ABBREVIATIONS

~ ---------- Approximately

aaMΦ ---------- Alternatively activated macrophage

ACh ---------- Acetylcholine

ANOVA ---------- Analysis of variance

ATP ---------- Adenosine triphosphate

Ca^{2+} ---------- Calcium

$CaCl_2$ ---------- Calcium chloride

cm ---------- Centimeter

DAB ---------- 3,3´-diaminodbenzidine

DAG ---------- Diacylglycerol

dH2O ---------- Distilled water

ELISA ---------- Enzyme-linked Immunosorbent Assay

EIS ---------- Enteric immune system

ENS ---------- Enteric nervous system

EPSP ---------- Excitatory postsynaptic potentials

GI ---------- Gastrointestinal

GPCRs ---------- G protein-coupled receptors

H_2O_2---------- Hydrogen peroxide

H&E ---------- Haematoxylin and eosin

HRP ---------- Horse radish peroxidase

Ig ---------- Immunoglobulin

IPANs ---------- Intrinsic Primary Afferent Neurons

IPSP ---------- Inhibitory postsynaptic potentials

JAK ---------- Janus tyrosine kinases

K^+ ---------- Potassium ions

KCl ---------- Potassium chloride

KH_2PO_4 ---------- Potassium hydrogen orthophosphate

KOH ---------- Potassium hydroxide

L3 ---------- Third stage larvae

IL ---------- Interleukin

IL-4Rα ---------- Interleukin-4 receptor alpha

IL-13Rα1 ---------- Interleukin-13 receptor alpha-1

LPS ---------- Lipopolysaccharide

M---------- Molar

$M_{\#}$ ---------- Muscarinic

MΦ ---------- Macrophage

mAChR ---------- Muscarinic acetylcholine receptor

$MgSO_4$ ---------- Magnesium sulphate

MHC ---------- Major histocompatibility complex

MLC ---------- Myosin light-chain

mM ---------- Millimolar

mN ---------- Millinewton

mRNA ---------- Messenger ribonucleic acid Na^+ Sodium ions

nAChR ---------- Nicotinic acetylcholine receptor

NaCl ---------- Sodium chloride

$NaHCO_3$ ---------- Sodium hydrogen carbonate

N. brasiliensis ---------- *Nippostrongylus brasiliensis*

NK-1 ---------- Neurokinin-1

OCT ---------- Organic cation transporter

PAS ---------- Periodic acid Schiff

PBS ---------- Phosphate buffered saline

PBST ---------- Phosphate buffered saline with tween

p.i. ---------- Post-infection

PNP ---------- 4-Nitrophenylphosphate

s.c ---------- Subcutaneous

SEA ---------- Soluble egg antigen

SEM ---------- Standard error of the mean

S. mansoni ---------- *Schistosoma mansoni*

SMC ---------- Smooth muscle cells

STAT ---------- Signal Transducer and activator of Transcription

SP ---------- Substance P

T_H2 ---------- T helper 2

TMB ---------- 3.3', 5.5'-tetramethylbenzidine

TNF ---------- Tumor necrosis factor

PUBLICATION

The following publication is based on studies that are described in this dissertation

Marillier RG, **Brombacher TM***, Benjamin Dewals, Mosiuoa Leeto, Dhirendra Govender, William GC Horsnell , Frank Brombacher (2010). **IL-4Rα-responsive smooth muscle cell increase intestinal hypercontractility and contribute to resistance during acute *Schistosomiasis*.** *Amer J Physiol Gastrointest Liver Physiol.* **298 (6): G943-951**

*** (joint first author)**

Front page: University of Cape Town logo

Abstract

Interleukin-4 receptor alpha (IL-4Rα) signaling, mediated by the ligands IL-4 and IL-13, is important for effective host protection during murine *Nippostrongylus brasiliensis* (*N. brasiliensis*) and *Schistosoma mansoni* (*S. mansoni*) infection. Among other cell types, IL-4Rα responsive smooth muscle cells influence immunological responses and are needed for host protection during *N. brasiliensis* and *S. mansoni* infection. This was shown in infected smooth muscle cell-specific IL-4Rα deficient (SM-MHC^{cre}IL-4Rα$^{-/lox}$) mice, which lead to delayed *N. brasiliensis* worm expulsion or *S. mansoni* egg retention. This was associated with reduced protective immune responses, confirmed in this study. As IL-4 and IL-13 are able to induce smooth muscle cell hypercontractility—contributing to worm expulsion—the ability of smooth muscle cells to contract in response to pharmacological stimuli in comparative studies with SM-MHC^{cre}IL-4Rα$^{-/lox}$, global IL-4Rα$^{-/-}$, and heterozygous control IL-4Rα$^{-/lox}$ mice was further investigated in this study. For this purpose, an *in situ* contraction method was established, which allowed to measure contractile forces from intestinal tissues in response to varying acetylcholine (ACh) concentrations, using an organ bath/powerLab^{TM} system.

Several methods for studying tissue contractile responses to a pharmacological stimulus were investigated for suitable use in mice strains employed in this project, namely:

- The cumulative method (a single piece of intestine tissue was stimulated with various concentrations of ACh with no resting intervals between stimulation)
- The single tissue/single concentration non-cumulative method (each individual piece of intestine was stimulated with a different concentration of ACh)
- The single tissue/various concentrations non-cumulative method (*sv.*non-cumulative method) where a single piece of intestine tissue was stimulated with various concentrations of ACh, coupled to 10 minutes resting intervals.

Optimization studies in non-infected and infected mice revealed that the *sv.*non-cumulative method was the most stable and reliable method for this purpose. Moreover, as tissue weight differences were apparent due to the different

histopathology between infected mouse strains, the weight was incorporated into the calculation of contractile tension. In the global absence of IL-4Rα during infection with *N. brasiliensis* or *S. mansoni*, contractile tension to ACh was impaired compared to infected control mice. The absence of IL-4Rα on smooth muscle cells resulted in reduced contractile tension in infected mice. From these results it is concluded that IL-4Rα-responsive smooth muscle cells increase intestinal hypercontractility and therefore contribute to resistance by enhancing worm expulsion during acute *N. brasiliensis* and egg transfer during *S. mansoni* infection.

General Introduction

(Part 1)

GI.1.1 Parasitic helminths

Prevalence of Helminth Infection

Over one third of the world's population is estimated to be infected with soil-transmitted parasitic helminths (Bethony et al., 2006; Colley et al., 2001; Hotez et al., 2006; McKenzie et al., 1998). These are generally identified as any parasitic worm such as nematodes, trematodes (flat worms), and cestodes (tape worm) (Hugot, 2001). These parasites infect their host via infective stage larvae found in moist soil [Figure 1.0]. Hookworm *Necator americanus* (*N. americanus*) belongs to the family of strongylid parasites that originate from the phylum Nematoda (Dorris et al., 1999). Nematodas are parasitic to vertebrates (e.g. human beings and rodents), and fall under the category of pseudocoelomates as helminths in the animal world (Hugot, 2001). They are unsegmented worms characterized by elongated, cylindrical bodies that often narrow on both ends (Hugot, 2001). Parasitic flatworms of the trematoda class, belonging to the schistosomatidae family of helminths, are identified by their thick outer cuticles. They have one or more hooks that attach to the host's tissue for feeding purposes. Some of the trematodes cause diseases like schistosomiasis in humans, primarily in tropical regions (Snyder, 2000). These parasites cause gastrointestinal (GI) infection (Bethony et al., 2006; Hotez et al., 2006) in more than half of the world's population.

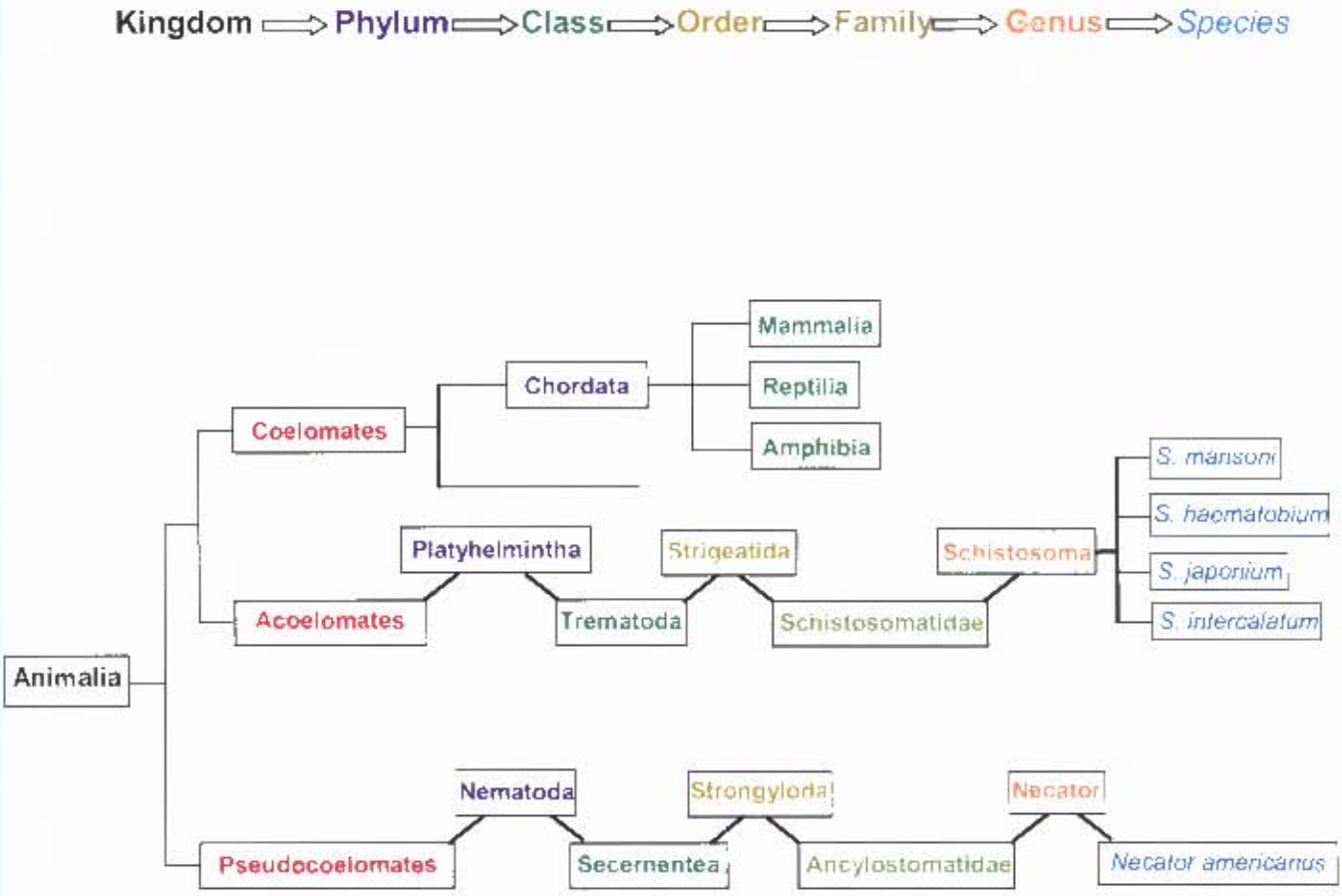


Figure 1.0

Helminths in the animal kingdom

A general classification of helminths: demonstrating origins of *Schistosoma mansoni* and *Necator americanus*. (Adapted from Hugot, 2001; Snyder, 2000 and Dorris et al., 1999).

Necator americanus

N. americanus is reported to be the most prevalent nematode infection both globally (Diemert et al., 2008) and in sub-Saharan Africa (de Silva et al., 2003; Hotez et al., 2009). Approximately two hundred million people in sub-Saharan Africa are estimated to be infected with hookworms (Diemert et al., 2008; Hotez et al., 2009) [Figure 1.1]. This high prevalence is associated with poor sanitation, limited supply of clean running water, and restricted availability of public health care (de Silva et al., 2003). Hookworm pathology has been correlated to increased morbidity rates (Hotez et al., 2008), cases of anemia (Colley et al., 2001), malnutrition, as well as altered development in children (de Silva et al., 2003) and susceptibility to other infections (Chenine et al., 2005). Hookworm attaches orally to the host's intestine and feeds on the blood, ultimately leading to intestinal blood and protein loss in proportion to worm burden (Diemert et al., 2008; Hotez et al., 2008). Clinical disease from hookworm infection is typically a result of the rate of host's blood loss surpassing their nutritional reserves, leading to an increased risk of anemia due to iron deficiency (Diemert et al., 2008). Children and pregnant mothers tend to be the most at risk from this pathology due to their limited iron reserves. This nutritional strain is also associated with impaired cognitive development in children (de Silva et al., 2003).

Hookworms such as *N. americanus* infect an individual when infective third-stage larvae (L3) penetrate unprotected skin, finding their way to the lymphatics via subcutaneous venules in order to access the blood circulation. From the pulmonary capillaries they penetrate lung alveolae, making way through the epiglottis where they are eventually coughed up and swallowed into the GI tract and adult worms develop (Bethony et al., 2006; Diemert et al., 2008). Approximately five to nine weeks are required for larvae to develop into egg-laying adults from the onset of penetration, and the parasite is able to dwell in the host's proximal small intestine attached to the mucosa for up to five years, feeding on blood as well as other nutrients (Bethony et al., 2006; Diemert et al., 2008).

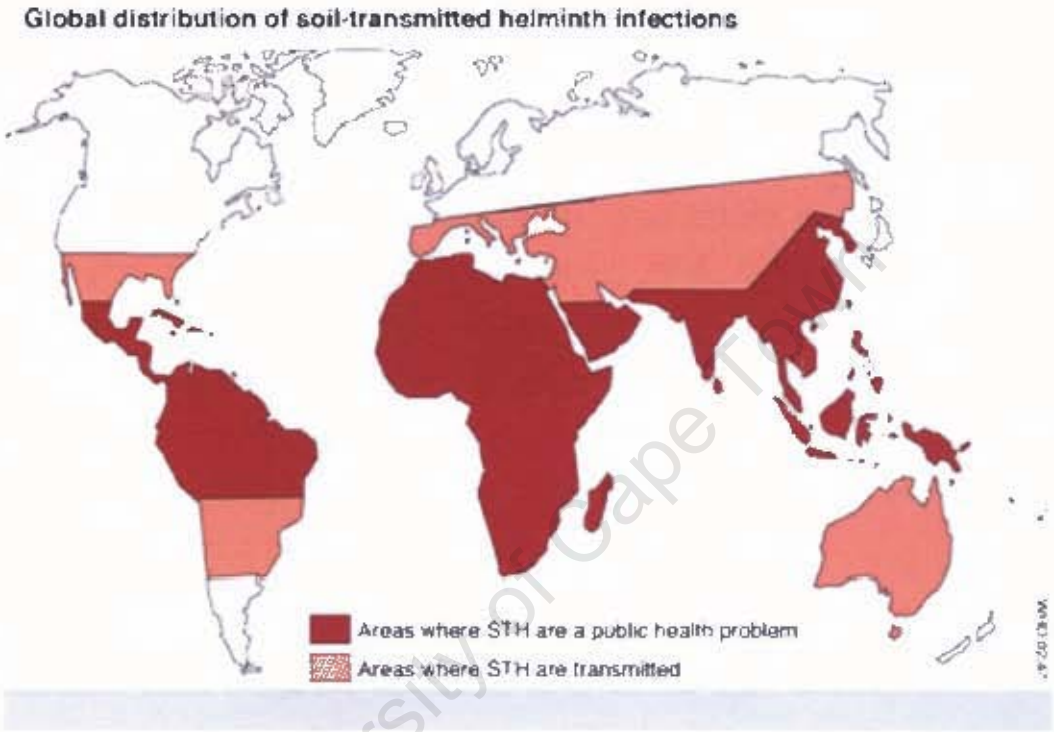


Figure 1.1

A geographic distribution of helminths

Areas where soil transmitted helminths are considered a public health are shaded red, and those where soil transmitted helminths are transmitted are shaded pink. (Adapted from World Health Organization).

Such a long lasting effect by *N. americanus* is associated with their secretion of immunomodulatory products which impair host immunity to the parasite. This then allows the parasite to invade the host's submucosal layer while ingesting villous tissue and sucking host's blood (Colley et al., 2001; Diemert et al., 2008). In order for the cycle to continue, adult worms mate in the intestine where females produce thousands of eggs daily. These eggs are then expelled from the host's body along with the feces (Bethony et al., 2006). In cases of poor sanitation and lack of latrine services, the expelled eggs will hatch in moist soil, developing into rhabditiform larvae which will go through several molting stages before becoming infective L3 that migrates to either higher ground or grass in search of a host. A murine model of hookworm is *Nippostrongylus brasiliensis* (*N. brasiliensis*) and has been studied extensively (Farid & Horii, 2009; Giacomin et al., 2008; Jones et al., 2009; Knott et al., 2009; Mearns et al., 2008; Zhao et al., 2009) [Figure 1.2]. Immuno-competent wild-type mice clear parasite infection at approximately ten days after infection, with day seven post-infection as the peak of parasite burden (Barner et al., 1998). These parasites feed on host tissue where they cause direct damage from worm movement and feeding (Symons & Fairbairn, 1962).

Drug treatment programs have been put in place to combat disease burden where benzimidazole class drugs are administered to school aged children either annually or semi-annually with an attempt to reduce infection intensity to levels that are not associated with disease (Diemert et al., 2008). Set backs that have been encountered include the fact that following de-worming, re-infection occurs due to unhygienic living conditions that include lack of clean running water for drinking and cooking (Savioli et al., 2004; Sow et al., 2008), leading to a continuous need for treatment that does not always reach the remote rural areas of developing countries where they are needed. Vaccines that may provide long-term protection, hence preventing hookworm disease have been proposed, and understanding the immunobiology of hookworm infection would aid in this regard.

Figure 1.2

Life-cycle of hookworm or murine N. brasiliensis *species*

N. brasiliensis Infects its host when infective stage 3 larvae enter the body via either the skin or mouth. They migrate to lungs where they are coughed up and swallowed. Upon reaching the small intestine, mature females produce eggs that get excreted with feces and hatch into larvae. (Adapted from World Health Organization).

Schistosoma mansoni

Schistosoma mansoni (*S. mansoni*) is a digenetic trematode that needs a minimum of two hosts to develop during its life-cycle. It causes schistosomiasis or bilharzia, affecting approximately two-hundred million people (Hotez et al., 2009). Infection causes liver damage characterized by granuloma formation (Leeto et al., 2006), as well as stunted growth, reduced fitness, and altered cognitive development in children (Hotez et al., 2009). Adult schistosomes are capable of residing in the host for many years, despite the host's competent immune responses. Schistosomiasis has been linked to morbidity which may be dependent on the type of immune response the host produces, along with the effect of the activated immune response on the formation of granuloma (Cheever et al., 2000; Dunne & Pearce, 1999).

Acute schistosomiasis is characterized by katayama fever that usually occurs prior to egg visibility in the host's stools, and tends to increase six-to-eight weeks following infection (Rabello, 1995). Unfortunately, due to numerous cases of re-infection in endemic areas for schistosomiasis, it is rare to come across cases of infection characterized by katayama fever as it tends to occur in individuals exposed to infection for the first time, and whose parents have no history of previous exposure either. This is because as individuals are likely to be sensitized to schistosomes *in utero* via maternal infection (King et al., 1998; Malhotra, 1997).

Chronic schistosomiasis on the other hand tends to be more severe and possibly life-threatening. It is classified as a hepatosplenic disease characterized by periportal and hepatic fibrosis, as well as portal hypertension (Dunne & Pearce, 1999). The intensity of infection may determine how severe chronic schistosomiasis is, especially in children (Mohamed-Ali et al., 1999). There appears to be an age-dependent correlation with schistosomiasis infection, with a trend demonstrating that children tend to carry higher parasite burdens while adults have lower burdens (Butterworth et al., 1994). Because of possible unhygienic practices, cases of re-infection appear to be common among children than adults who still maintain lower parasite burdens than children when re-infected. Chemotherapy that has been employed to control schistosomiasis appears to be ineffective because of numerous re-infection cases as well as drugs themselves failing to reach those who are in dire need of them in the most rural endemic areas.

Because most of the individuals that are infected are asymptomatic, generally unaware of their status, helminth infections may be referred to as "silent killers". Trematode *S. mansoni* infects human beings when larvae (cercariae) from snails in the water penetrate the skin where they later transform into schistosomula [Figure 1.3]. After a few days, this parasite migrates through the blood stream into the lungs and intestine, where it matures into male and female adult worms (Pearce & MacDonald, 2002).

Figure 1.3

Life-cycle of the Schistosoma *species*

Schistosomatidae have more than one host (e.g. human beings and snails). Asexual reproduction occurs in the snails while sexual reproduction occurs in a human host. Infection is initiated when cercariae burrow into the host's skin. It eventually transforms into Schistosomula before entering the vasculature and migrating to the portal system. There they mature into adult worms. Female parasites within the vasculature release eggs that cross the membrane of the veins traversing the intervening tissue and intestine epithelium *en route* to the exterior (adapted from Pearce & MacDonald, 2002).

S. mansoni adults mate in the mesenteric venules where they produce eggs that require fresh water in order to release free swimming larvae (miracidia) that eventually penetrate snails as their intermediate host, and the cycle continues. *S. mansoni* has been characterized extensively (Abdu, 2009; Criscione et al., 2009; Duraes et al., 2009; Haas & Haeberlein, 2009; Tait, 2009; Taman & Ribeiro, 2009), and the life-cycle in mice is similar to that of human beings, with the chronic phase of infection determined after eight weeks.

GI.1.2 The enteric immune system response to helminths

The GI tract has an intrinsic immune system of its own called the enteric immune system that begins in the esophagus, extending to the anus with key roles in controlling most GI functions, especially movements and secretion. Micro-organisms (pathogens) such as bacteria, viruses, or multi-cellular parasites including helminths are able to invade the body and possibly multiply and destroy functional tissue. In order for the body to defend itself from some of these disease-causing pathogens, it developed several defense mechanisms such as protective surface, non-specific tissue defense, as well as specific immune responses.

Protective surface mechanisms

The skin serves as first line of defense against pathogens that may enter the body through breaches in either the skin or linings of the intestine. The intestine for instance is lined with mucus-secreting goblet cells on villi that are in constant gentle agitation, in turn expelling any possible pathogens. In case this type of defense fails, other types of defense mechanisms are activated.

Non-specific tissue defenses

The skin serves as an impermeable barrier to most organisms that usually gain access to the body when tissue is damaged, exciting a non-specific response known as inflammation. Inflammation can either be acute or chronic, with the aim being to excrete the pathogen and then regenerate lost tissue. In the case of acute inflammation, there is an increase in blood flow and neutrophils. This is followed by macrophages migrating to the injured area in order to phagocytose and kill the pathogen. Any tissue damaged in this process is gradually regenerated with the aid

of fibroblast proliferation and collagen deposition (Leeto et al., 2006; Wynn et al., 2004).

Specific immune responses

Upon recognition of an antigen, the immune system is activated with a purpose of either neutralizing or destroying the antigen. Lymphocytes have been shown to play a key role in the immune response, with dependence on phagocytic cells like macrophages for antigen presentation. Neutrophils and macrophages from the non-specific defense system are also recruited and amplified for final antigen destruction.

For effective protection of the host from helminths, the host needs to generate a T helper 2 (T_H2)-type response with T_H2 cytokines interleukin-4 (IL-4) and interleukin-13 (IL-13) that are important to resolve the infection (Barner et al., 1998; McKenzie et al., 1998; Urban et al., 1998). IL-4 and IL-13 are closely related cytokines with pleiotropic immune functions that are mediated through a shared IL-4Rα subunit by their respective receptors (Brombacher, 2000) present on hematopoietic and non-hematopoietic cells (Nelms, 1999). IL-4 is capable of signaling through the type I receptor comprised of the IL-4Rα chain and the common gamma (γc)-chain, as well as the type II receptor comprised of the IL-4Rα chain and the IL-13Rα1 chain. IL-13 on the other hand signals via the type II receptor (Hilton, 1996). IL-4 is a B-cell antibody Isotype switch factor to IgG1 as well as IgE (Brombacher, 2000). Murine T- and B-cells do not express a functional type II receptor, whereas human B cells are responsive to IL-13 (Zurawski & de Vries, 1994). The binding of IL-4 or IL-13 to the IL-4Rα activates janus kinase (JAK) family members to initiate phosphorylation. This is followed by dimerization of STAT-6 that migrates to the cell nucleus, binding to various gene promoters (Sholl-Franco, 2009) [Figure 1.4].

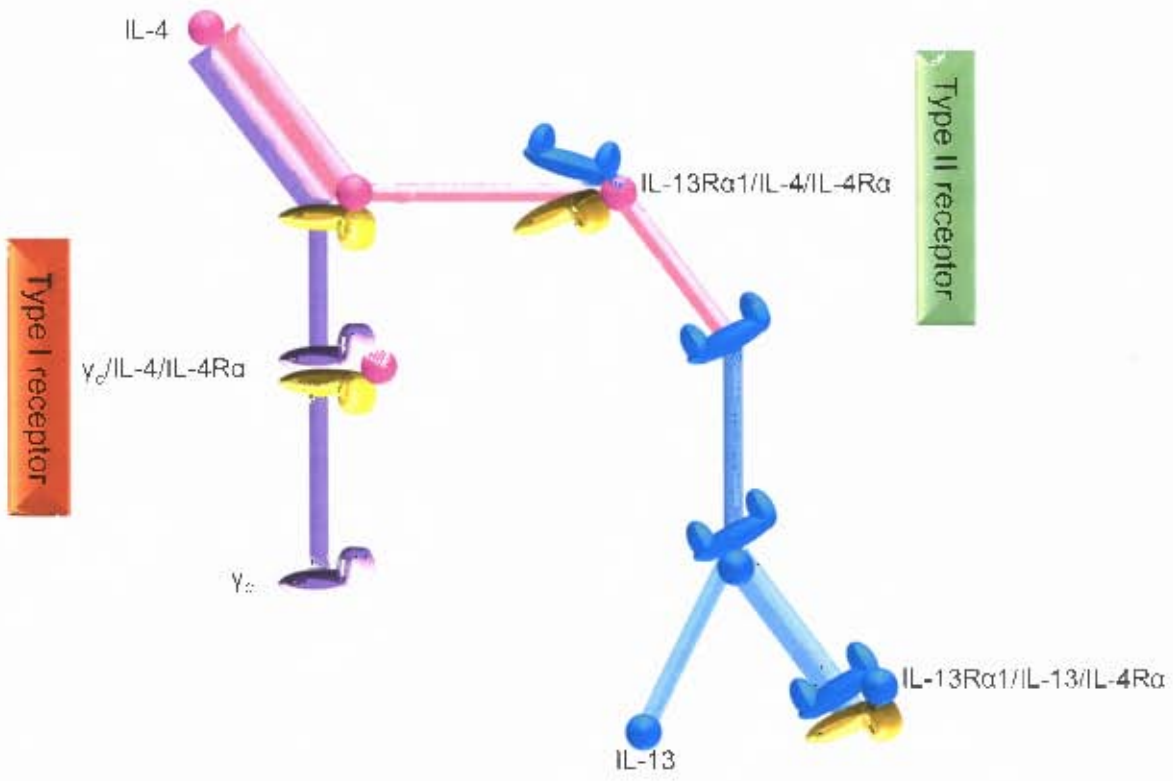


Figure 1.4

Schematic representation of IL-4 and IL-13 receptor complexes

IL-4 interacts with Type 1 (IL-4Rα and gamma chain (γ_c)) as well as with Type 2 (IL-4Rα and IL-13Rα-1) receptor complexes. [**Pink ball**: IL-4, **Pink line**: IL-4 pathway, **Blue ball**: IL-13, **Blue line**: IL-13 pathway, **Purple line**: IL-4 pathway, **Yellow** T-bar: IL-4Rα, **Blue bar**: IL-13Rα1, **Purple T-bar**: gamma chain]. (Adapted from Brombacher, 2000; Hershey, 2003).

Enteric Immune System T_H2-type protection during N. brasiliensis *Infection*

Coughed up and swallowed *N. brasiliensis* L3 travel to the small intestine where they invade intestinal epithelia, dwelling in the sub-mucosa for approximately seven days. Upon entry into the sub-mucosa, L3 release antigens that are presented to T cells by peptide-MHC complexes found on surfaces of antigen-presenting cells in gut-associated lymphoid tissues that are distributed throughout the GI tract. The activated T cells proliferate and differentiate into $CD4^+$ T_H1 and T_H2, thus activating T_H2 effector cells. Activated T_H2 cells produce and release cytokines IL-4, IL-13, IL-5, and IL-9 among others, in turn leading to a T_H2-type response characterized by B-cell immunoglobulin class-switching to IgG1 and IgE—a classical marker for T_H2-type response (Barner et al., 1998; Fallon et al., 2002), while activated effector cells migrate to the site of invasion for antigen destruction (Fallon et al., 2002). IgE response promotes mast cell degranulation and has effects on non-bone marrow derived cells such as intestinal cells to increase their sensitivity to mediators released by activated mast cells (Finkelman et al., 2004).

IL-4 and IL-13 produced in the intestinal mucosa by activated T_H2 cells leads to enhanced permeability of the epithelial cells as well as increased smooth muscle cell contractility (Zhao et al., 2003). This activity accentuates the ability of the larvae to be excreted from the sub-mucosa and back into the lumen. After seven days post-infection, L3 re-enter the lumen where they mature into adult male and female nematodes. Goblet cell hyperplasia is observed, and mucus production/secretion coupled with enhanced smooth muscle contractility leads to a "weep and sweep" response that aids in worm expulsion from the intestine. A strong host immune response results in (i) intestinal inflammation (Akiho et al., 2002), (ii) secretion of large amounts of mucus from goblet cells hyperplasia (Miller et al., 1981) that inhibit worms' ability to move and feed (Lee & Biggs, 1990), and (iii) heightened enteric smooth muscle contractions (Zhao et al., 2003) for physical peristaltic worm expulsion from the host (Khan & Collins, 2006) [Figure 1.5]. Reduced worm expulsion has been found in mice that are deficient in STAT-6 (Urban et al., 1998), IL-4Rα (Barner et al., 1998; Urban et al., 1998) or IL-13 (McKenzie et al., 1998).

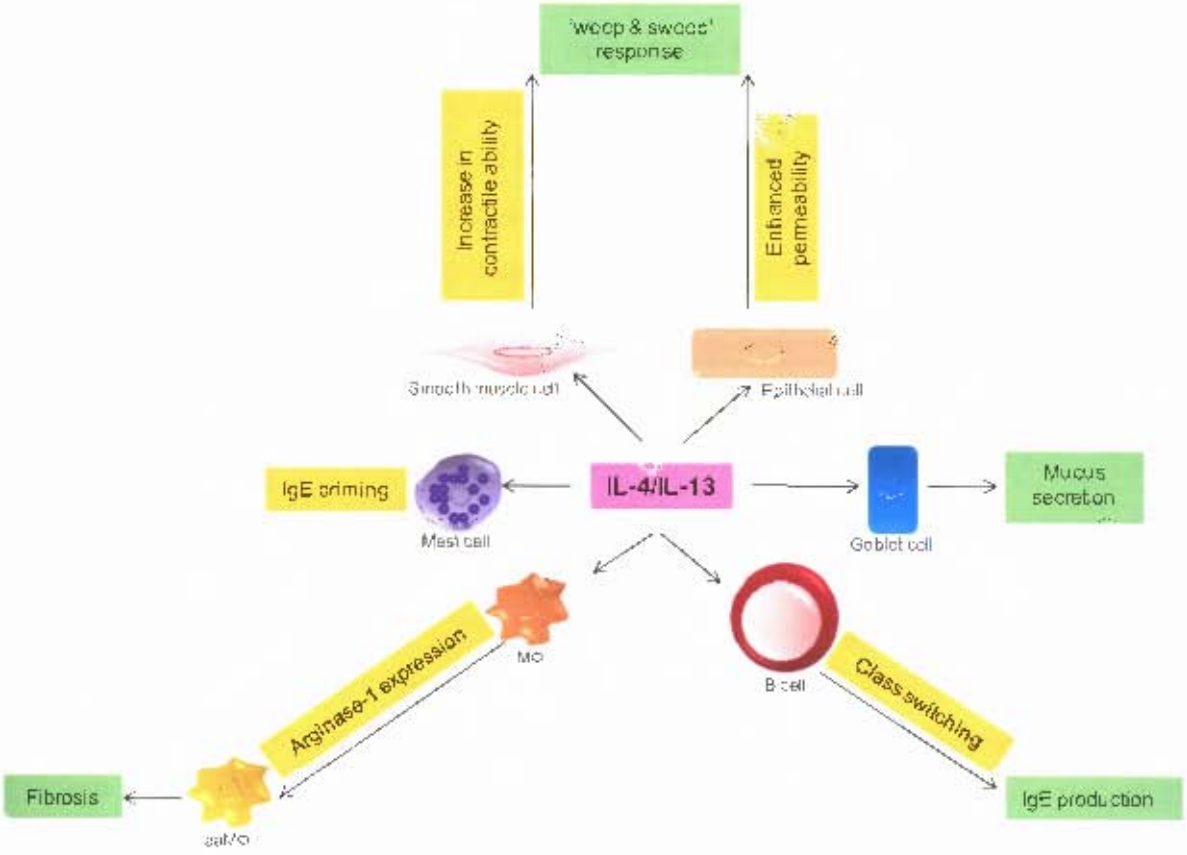


Figure 1.5

A schematic representation of IL-4 and IL-13 functions during helminth infection

IL-4 and IL-13 produced by T_H2 cells enhance mast cell development, increased intestinal permeability, elevated goblet cell mucus secretion, and increased smooth muscle cell contractility. Together, these responses enhance a "weep and sweep" response to intestinal helminths for host protection. [aaMΦ: alternatively activated macrophages, MΦ: macrophages]. (Adapted from Anthony et al., 2007).

Enteric Immune System T_H2-type protection during S. mansoni *Infection*

Unlike *N. brasiliensis* that produces an immediate T_H2-type cytokine response from $CD4^+$ T cells within 24 hours of infecting the host, the initial stages of the infective cercariae tends to activate T_H1-type responses in murine experimental models (Pearce & MacDonald, 2002) [Figure 1.6]. After approximately 5 weeks, adult worms produce eggs that are released into the vasculature, leading to a T_H2-type response against egg antigens (Herbert, 2004; Leeto et al., 2006). In this model of infection, IL-4 and IL-13 acting on macrophages lead to arginase-1 expression that activates alternatively activated macrophages (AAMΦ) (Herbert, 2004).

Following eight weeks post infection, murine models enter a chronic phase where eggs accumulate in the liver (Taylor et al., 2009). At this stage, mice lacking IL-4Rα die from inflammatory reactions to the parasite (Leeto et al., 2006; Marillier et al., 2008). In order for the host to be protected against both helminths (*N. brasiliensis* and *S. mansoni*), the individual requires successful signaling of IL-4 and IL-13 via IL-4Rα. During the acute phase of schistosomiasis in human beings, tumour-necrosis factor (TNF) expression in the plasma is elevated, and increased levels of IL-1 and IL-6 are noticeable in certain cells (de Jesus, 2002). As "disease" progresses, eggs produce antigen that induces a T_H2-type response that down-regulates but does not abrogate the production and function of pro-inflammatory cytokines (TNF, IL-1, and IL-6). The induced T_H2-type response is responsible for the production of cytokines that may be able to inhibit detrimental inflammatory mediators (Pearce & MacDonald, 2002).

GI.1.3 The enteric nervous system response to helminths

In addition to having an enteric immune system, the GI tract also has an intrinsic nervous system of its own called the Enteric Nervous System (ENS) that begins in the esophagus and extends all the way to the anus. This arrangement suggests that the intestine can perform as a "mini brain", functioning independently from the brain via the vagus nerve to control most GI functions, especially movements and secretion.

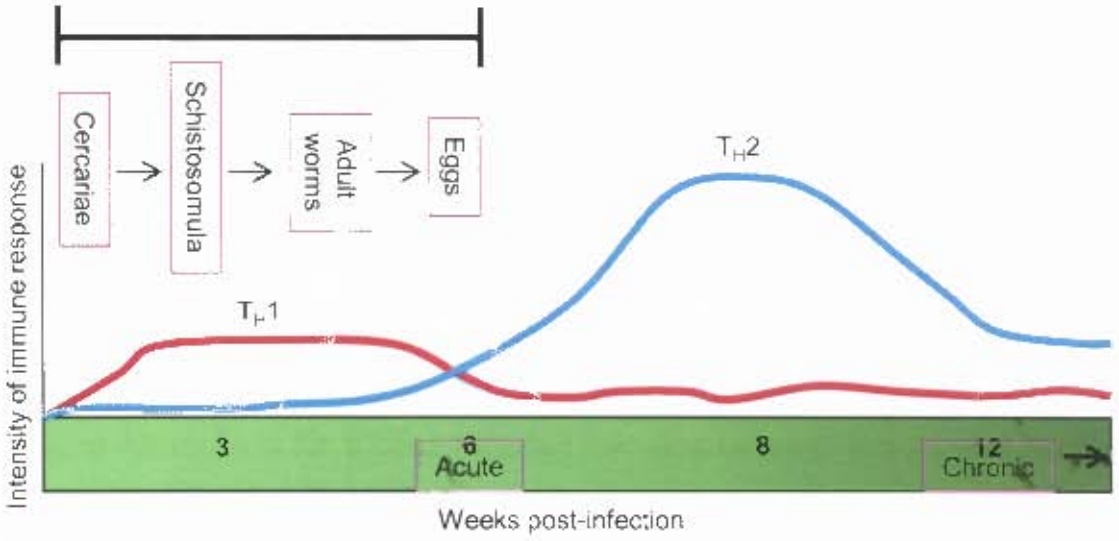


Figure 1.6

Immune response development during schistosomiasis

Immune response progresses through a minimum of three stages. The host harbors migrating immature parasites during the first 5 weeks, demonstrating a dominant T_H1-like response. Following 5 weeks post-infection, the parasites mature, mate, and produce eggs that enhance an altered immune response characterized by a decrease in the T_H1-like response and emergence of a strong T_H2 response. This T_H2 response is modulated after 12 weeks of infection, and granulomas that form around the eggs are reduced in size compared to earlier infection time-points. (Adapted from Pearce & MacDonald, 2002).

The enteric nervous system is composed mainly of two types of ganglia and appropriate connecting fibers (Dodd, 1991). The outer muscle layer has a group of neurons known as the myenteric plexus that lie between the longitudinal and circular muscle layers. They function to control the GI movements, with most excitatory fibers being cholinergic, secreting ACh. In the inner layer lies the submucosa containing the submucosal plexus, which is important for controlling secretion and blood flow [Figure 1.7].

In addition to the host's immune response, the enteric nervous system may play a role in worm expulsion by means of cholinergic driven intestinal contractions (Zhao et al., 2003), and innervations of mucus producing cells (Stead et al., 1987). It has been demonstrated that mice deficient of muscarinic neurotransmitter receptors (e.g. M_3 receptors) have reduced contractility in the smooth muscles of the intestine (Matsui et al., 2002), which may in turn lead to attenuated worm expulsion. Neuro-immune interactions have been reported following helminth infections. Substance P (SP), a neuro-peptide belonging to the family of tachykinins is expressed in both the central nervous system and the peripheral nervous system, including the GI tract, along with its receptor neurokinin-1 (NK-1R) (Koon, 2006). During schistosomiasis, SP has been noted to communicate with immune cells that play a role in pathology and disease progression (Weinstock, 2004). Specifically, it has been shown that SP controls the secretion of immunoglobin in granuloma cells of infected mice (Neil, 1991). Mast cells have been shown to interact with SP-containing neurons during *N. brasiliensis* infection (Bienenstock et al., 1987) suggesting neuro-immune interaction *in the GI tract in this model.*

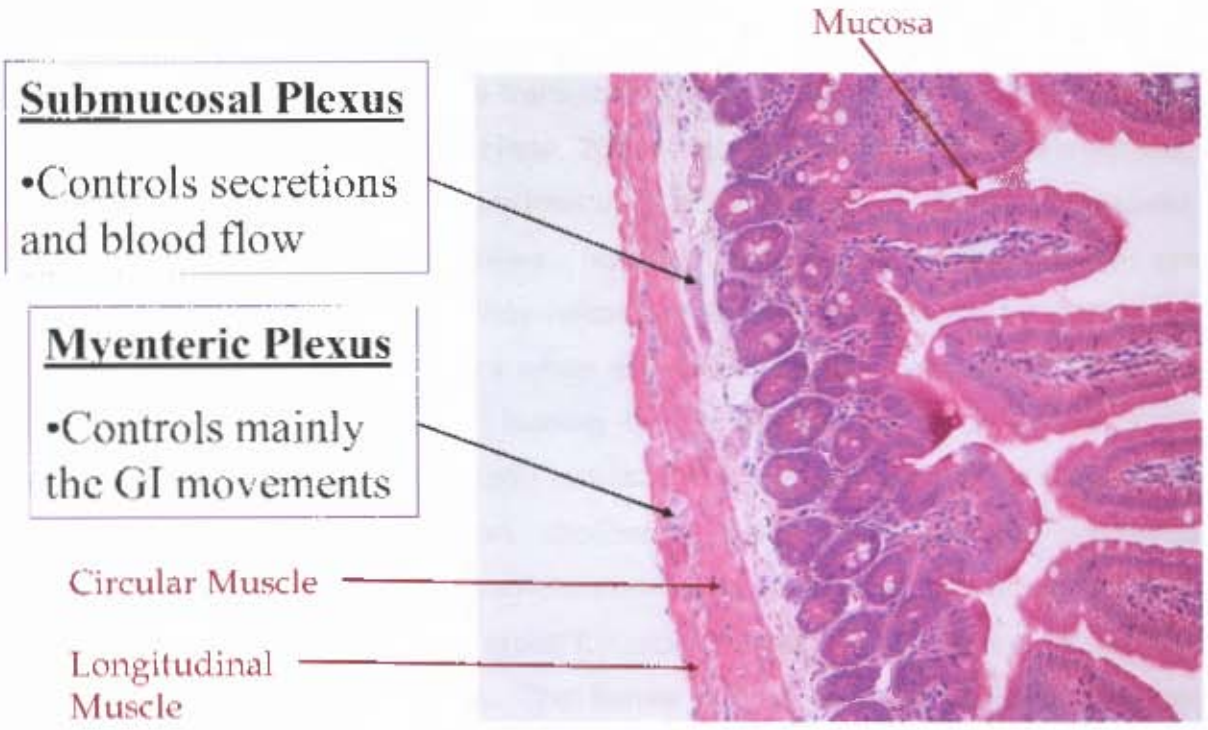


Figure 1.7

Schematic representation of the small intestine smooth muscle layer

Tissue sections were cut 10cm from the stomach pylorus of non-infected BALB/c mouse jejunum (H&E staining). Black arrows point to plexus areas containing ganglia, and red arrows point to the outer layer of the intestine (muscularis) and the inner layer (mucosae) with villi. (Picture taken by T. Brombacher, 2007).

GI.1.4 Acetylcholine

Acetylcholine (ACh) is a neurotransmitter contributing to the functions of both the central nervous system and the peripheral nervous system (Prado et al., 2002). It is synthesized by the enzyme choline acetyltransferase from choline and acetyl-CoA which are substrates, with choline transported from extracellular sites by a choline transporter into the cell (Okuda & Haga, 2000). Upon synthesis, ACh is transported into synaptic vesicles by a transvesicular proton gradient driven transporter (Parsons, 2000) where it accumulates. Neurons producing and releasing ACh are known as cholinergic neurons. They release ACh from the vesicles by exocytosis during depolarization which occurs when an action potential reaches the terminal button of a presynaptic neuron, leading to the opening of voltage-gated Ca^{2+} channels (Racke et al., 2006). Upon release, ACh may act on nearby target cells (post-junctional receptors) or on cholinergic nerve terminals (pre-junctional autoreceptors). The enzyme acetylcholinesterase terminates the action of ACh immediately following release in order for repolarization to take place, degrading ACh into choline and acetic acid. The former is then recycled back into the cell (Gwilt et al., 2007; Racke et al., 2006) via the high affinity choline transporter.

ACh synthesis and release can be both neuronal and non-neuronal [Figure 1.8]. In non-neuronal cells such as smooth muscle cells, lymphocytes, and macrophages, there are no storage vesicles for ACh, but rather ACh is released immediately following synthesis (Gwilt et al., 2007). The mechanisms involved in both the synthesis and release of ACh in non-neuronal cells is not clear, however, there is evidence that ACh is transported via active transport by the aid of Organic Cation Transporter family (OCTs) (Lips et al., 2005).

GI.1.5 Acetylcholine Receptors

ACh is known to bind to both nicotinic (nAChR) and muscarinic (mAChR) receptors, where ACh binds nicotinic receptors immediately, evoking fast excitatory postsynaptic potentials (EPSP), triggering an action potential in postganglionic cells (Gwilt et al., 2007). These receptors are transmitter-gated cation channels and are found on target muscles or cells at junctions (Racke et al., 2006). Following release, ACh binds nAChR leading to the opening of ion channels in target muscles or cells.

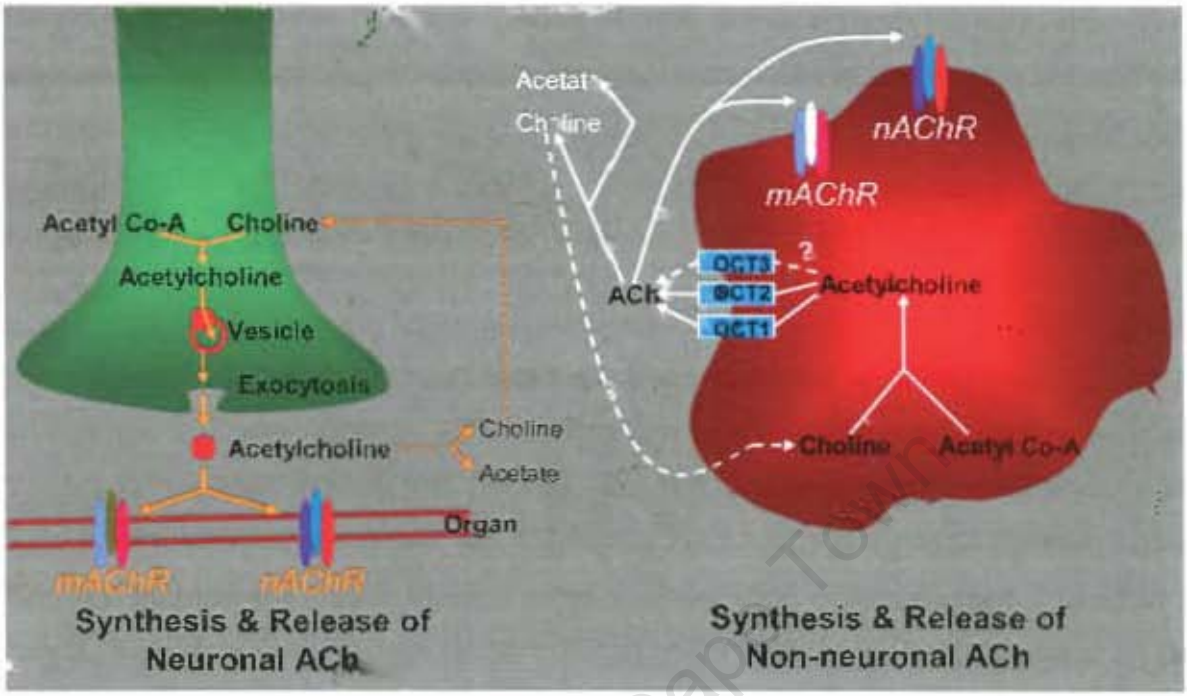


Figure 1.8

A schematic representation of acetylcholine synthesis in both neuronal and non-neuronal cells

Acetylcholine (ACh) is synthesized and released in both neuronal and non-neuronal cells (e.g. epithelial cells, smooth muscle cells, as well as in various immune cells like macrophages and lymphocytes). There are two major types of ACh receptors, namely muscarinic (mAChR) and the nicotinic (nAChR). Neurons, as well as T and B lymphocytes store ACh in vesicles, whereas most non-neuronal cells do not store ACh, but rather release it directly following synthesis. Similarities between neuronal and non-neuronal ACh includes that they both produce ACh and act on similar receptors. They differ in that neuronal ACh is released via vesicles during exocytosis and is stored in vesicles until calcium influx. Non-neuronal ACh on the other hand depends on an active transport system for release, and is not stored because it is only produced when needed. [**nAChR**: nicotinic acetylcholine receptors, **mAChR**: muscarinic acetylcholine receptors, **OCT**: organic cation transporter]. (Adapted from Racke et al., 2006 and Gwilt et al., 2007).

This process allows positive ions to pass through (mostly Na^+ and K^+), resulting in a change of membrane potential and thus signal transmission (Racke et al., 2006). mAChR demonstrate a slow onset of excitation, and require repetitive activation of the preganglionic nerve before evidence of their activation can be detected. They are metabotropic receptors (G-protein coupled) with ability to evoke both the opening and closing of ion channels leading to very slow EPSPs and inhibitory postsynaptic potentials (IPSPs) (Racke et al., 2006). They generally involve second-messenger systems instead of directly opening ion channels, activating a cascade of intracellular pathways.

Five subtypes of muscarinic receptors have been identified and classified as M_1—M_5 (Uchiyama & Chess-Williams, 2004). They are expressed in most cell types. "Odd numbered" muscarinic receptors (M_1, M_3, and M_5) tend to couple to G-proteins of the $G_{q/11}$ family while others prefer the $G_{i/o}$ family (Uchiyama & Chess-Williams, 2004). $G_{q/11}$ are guanine nucleotide binding proteins stimulating phospholipase C in order to generate a second-messenger inositol triphosphate (IP3) which is responsible for the induction of calcium release from intracellular stores, as well as diacylglycerol (DAG), causing extracellular calcium influx (Caulfield, 1993). Direct contractile responses in the GI tract appear to the mediated exclusively by M_3 receptors, even though the M_2 receptor subtype is more abundant than the M_3 in the GI tract (Michel & Whiting, 1988).

GI.1.6 Intrinsic Primary Afferent Neurons

Intrinsic primary afferent neurons (IPANs) of the enteric nervous system are found among other peripheral neurons (Furness, 2004), with their cell bodies found embedded in walls of the intestine. These neurons are identified as large and oval, with several long axonal dendrites that may innervate intestinal lumen. They tend to synapse with other neurons including enteric interneurons, motor neurons, as well as with other IPANs (Brehmer, 2006). This type of morphology is referred to as Dogiel type II, and therefore IPANs are also known as Dogiel type II neurons (Furness et al., 2004). The reason these neurons are referred to as primary is that they are the first neurons in the reflex pathways. Even though they convey information to visceral receptors, they may not require a stimulus to be activated, hence the term afferent

being used to refer to them as they do not necessarily "sense" stimuli, nor do they evoke sensation, but carry information to reflex centers (Furness et al., 2004).

IPANs have their cell bodies in the myenteric plexus and the submucosal plexus, with their dendrites terminating in the mucosa. They have been called intrinsic because unlike the vagal and the spinal primary afferent neurons, IPANs do not innervate the brain nor the spinal cord, but rather are exclusive to the intestine, conducting functions independent of the brain (Brehmer et al., 2004), giving the intestine a chance to function as a "mini-brain". Myenteric IPANs respond to chemical alterations in the lumen as well as to any changes that may take place in the external muscle layer of the intestine, whereas submucosal IPANs are more inclined to mechanical changes that may take place in the mucosa, along with chemical alterations in the lumen (Furness, 2004).

GI.1.7 Smooth Muscle Cells

Smooth muscle forms a large part of the GI wall, with individual smooth muscle cells being "spindle shaped". They are characterized as being long and having narrow width, generally being wide in the middle and tapered at each end, with the nuclei occupying the widest part of the cell. These cells overlap each other in order to form a sheet via gap junctions formed from plasma membranes and adjacent cells for communication and tissue cohesiveness. The wide part of one cell lies against the tapered end of the other, and the general purpose of smooth muscle is contraction of organs such as the GI tract (Webb, 2003). This type of muscle is capable of developing isometric tension per cross-sectional area. Actin filaments of smooth muscle are arranged through connections to dense bodies that contain α-actinin and also have intermediate filaments that contain proteins desmin and vimentin. Fibers of multi-unit smooth muscle are generally innervated by both the sympathetic and parasympathetic nerve fibers, and each unit responds independently from each other during nerve stimulation. The nerve stimulation of smooth muscle causes a membrane depolarization. In smooth muscle, this excitation-contraction coupling is known as electromechanical coupling (Somlyo, 1968). In this case Ca^{2+} permeates from extracellular space into the cell.

An excitation mechanism that is independent of membrane potential change, but is rather based on the activation of receptors by drugs and hormones—resulting in

muscle contraction—is termed pharmacomechanical coupling (Somlyo, 1968). This type is characterized by Ca^{2+} being released from the sarcoplasmic reticulum of the cell (Webb, 2003). Both of these mechanisms alter tonic contraction to maintain organ dimensions against imposed load, as well as tension development and muscle shortening. Upon Ca^{2+} binding to protein calmodulin, the complex activates myosin to bind to actin leading to smooth muscle cell (SMC) contraction (Webb, 2003). Upon contraction, SMCs shorten enabling propulsion of intestinal contents; however, cell shortening tends to vary the diameter of the intestine in order to regulate the flow of the contents.

SMCs receive neural connections from the autonomic nervous system and their contractility is generally dependent upon hormones and local chemical signals. Depending on changes in both load and length, SMCs develop tonic and phasic contractions. Despite the type of stimulus acting on the SMCs, cross-bridges cycling between actin and myosin are used for tension development, with Ca^{2+} initiating contractions (Webb, 2003). Contraction may be controlled by receptor and stretch activation of the contractile proteins myosin and actin. Myosin light-chain kinase (MLC-kinase) phosphorylates the light chain myosin, allowing myosin to interact with actin. Energy released from ATP by myosin ATPase activity enables the cycling of myosin cross-bridges with actin for contraction to occur (Malmqvist & Arner, 1999). In the absence of external stimuli, phosphorylation of the light chain of myosin is maintained at a low level, resulting in smooth muscle tone that can vary in intensity. In order for the smooth muscle to relax, a decrease in intracellular Ca^{2+} concentration along with an increase in MLC phosphatase activity must occur (Morgan, 1990).

GI.1.8 Objective of the project

The aim of this study was to extensively investigate the mechanisms through which the cholinergic, nervous, and immune systems of the intestinal smooth muscle interact, aiding in nematode *N. brasiliensis* expulsion. The presence of either IL-4 or IL-13 is associated with increased smooth muscle cell contractility as cells pretreated with these cytokines contract more than non-treated controls in the presence of carbachol (Akiho et al., 2002; Akiho et al., 2005), exhibiting the role of immune products on neurotransmitter enhanced hypercontractility. Using wild-type and

STAT-6$^{-/-}$ mice treated with *N. brasiliensis*, Zhao et al., 2003 demonstrate that stimulatory responses to ACh are enhanced in wild-type mice compared to their STAT-6$^{-/-}$ counterparts. These responses were attenuated but not abrogated in STAT-6$^{-/-}$ mice, suggesting that neurotransmitter effects on enhanced contractile ability of smooth muscle cells is partly dependent on immunological pathways. In the absence of M_2 and M_3 receptors in the intestine ileum longitudinal smooth muscle fails to respond to carbachol, even at concentrations that evoke maximal contractions in wild-type mice, suggesting the direct effects of ACh on smooth muscle contractility (Matsui et al., 2002).

Basically these studies demonstrate that hypercontractility of the smooth muscle cells is dependent on both immune (IL-4 and IL-13) and non-immune (acetylcholine/carbachol) products. The role of IL-4Rα expression on smooth muscle cells hypercontractile responsiveness was investigated in this study. Horsnell et al., 2007 demonstrate that IL-4Rα deficient mice show significantly lower levels of M_3 receptor mRNA expression compared to their wild-type controls. This was the first report recorded of IL-4Rα and M_3 receptor mRNA positive correlation *in vivo*.

The objective of this project was to build on results shown by Horsnell et al., 2007, establishing a link between IL-4Rα and M_3 receptors in enhanced smooth muscle cell contractility. The aim was to explore neuro-immunological interactions involved in *N. brasiliensis* expulsion using small intestine isolated tissue.

Specific objectives

1. Physiological studies to examine contractile effects of IL-4Rα in both infected and non-infected tissue using neurological agonists
2. To investigate the effects of IL-4Rα expression on both immune and non-immune cell populations
3. To study cytokine responses and native levels of immune and non-immune products in the presence of IL-4Rα

Materials and methods

Materials and methods

Mice

These experiments were conducted on both male and female BALB/c mice as well as on C.Cg-IL4Ra^{tm1Fbb}/IL4Ra^{tm2Fbb}Tg(Myh11-cre)5013Gko mice (SM-MHC^{cre}IL4Rα$^{-/lox}$ the synonym used in this project) on a BALB/c background that were generated and characterized as previously described (Horsnell et al., 2007). Cre IL-4Rα-deficient negative (IL-4Rα$^{-/lox}$) mice were used as their littermate controls (Herbert, 2004), along with IL4Ra^{tm1Fbb}/IL4Ratm1Fbb mice (IL-4Rα$^{-/-}$ the synonym used in this project) (Mohrs et al., 1999). All mice were aged between 6 and 8 weeks, and were kept in individually ventilated cages under specific pathogen-free conditions within the biomedical animal faculty of the Health Science Faculty, University of Cape Town (UCT). All experiments were performed in accordance to guidelines by the Animal Ethics Research Board of UCT.

Nippostrongylus brasiliensis larvae infections

N. brasiliensis infections were conducted as previously described (Befus et al., 1979). *N. brasiliensis* was passaged through 8 weeks old Wistar rats by inoculating them with ~5000 3rd stage larvae (L3)/ rat in 0.65% saline (a kind gift from Klaus Erb, Wurzburg, Germany). Rats were injected subcutaneously (s.c.) with live L3 in the neck using 18x1½ G needles (Strecan®, B. Braun, Melsungen). At days 6, 7, and 8 p.i. fecal pellets were collected in 250µg/ml Amphotericin B fungizone (Lot# 3079308, Invitrogen Corporation) treated dH_2O. The emulsified fecal pellets and Proanalysi charcoal (Merk, Darmstadt) were then mixed to a paste and placed on moist Whatman® filter papers, where eggs were allowed to hatch and larvae subsequently migrate to the edge of the filter paper. Larvae were harvested into 0.65% saline after a week and used to infect mice at ~750 L3 per mouse, administered s.c. using 21x1½ needles (Strecan®, B. Braun, Melsungen).

Quantification of intestinal *N. brasiliensis* worm burden

Parasite burden was determined as previously described (Barner et al., 1998). Individual whole small intestine tissue samples per mouse were incised longitudinally at the mesentery before being placed in CentriStar™ cap 15ml Corning® centrifuge tubes (Corning, NY) containing 0.9% saline and agitated at 37°C in a 5% CO_2

incubator for 4 hours and day 7 and 10 p.i. After worms dispersed out of the tissue, they were counted in Sterilin petri dishes (Staffs, UK) for parasite burden determination.

Quantification of intestinal *N. brasiliensis* egg burden

Analyses of fecal egg burden were performed using the modified floatation technique (Barner et al., 1998). Mice were inoculated with ~750 L3 s.c. The numbers of parasite eggs were determined for each mouse from fecal pellets collected at days 4, 7, 8, 9, and 10 p.i. Six to eight dry fecal pellets were collected in pre-weighed CentriStar™ cap 15ml Corning® centrifuge tubes (Corning, NY) in order to equalize sample size. 10ml of distilled water (dH_2O) was added to each tube and agitated at 4°C for approximately 16 hours. 1ml of sample was taken from each individual tube and transferred to a clean CentriStar™ cap 15ml Corning® centrifuge tube (Corning, NY), to which 9ml of saturated saline was added. This was done in order to reduce the amount of fecal debris that made it challenging to identify eggs. 1ml of sample was collected from the top of the meniscus where eggs float, and placed into a small Sterilin petri dish (Staffs, UK) for egg burden determination using an inverted microscope. Values obtained were averaged and normalized according to fecal weight.

Schistosoma mansoni live parasite infections

Anaesthetized (Anaket-V, Centaur Labs, RSA and 2% Rompun, Bayer, Germany) mice were infected with live Puerto Rican *S. mansoni* strain passaged through *Biomphalaria glabrata* snails (a kind gift from Andrian Mountford, York, UK). Snails were kept under a regulated light/dark cycle in conditioned water at 25°C and fed commercial fish flakes. Shaven mice abdomens were wiped with conditioned water on gauzes after restraining their movement. Cercariae (80, or 100 burden) shed from snails (Coligan, 1991; Herbert, 2004) were adjusted to required concentrations and pipetted into stainless steel rings that were placed on the abdomens, and removed after 1 hour. Following infections, mice were returned to their home cages and investigated at 8-9 weeks p.i. *[Infections conducted by Dr. Reece Marillier]*

Histological analyses

Intestine and liver tissue samples were fixed in 4% formalin in phosphate buffered saline (PBS) solution. Samples were embedded in paraffin and cut into 5-7µm sections and stained with haematoxylin and eosin (H&E) for inflammatory cells, periodic acid Schiff's (PAS) for mucus producing goblet cells, or chromotrope 2R and analine blue solution, counterstained with Wegert's hematoxylin for collagen detection. The number of PAS positively stained cells per 5 villi from 4 different jejunum sections per mouse were counted by light microscopy. Samples were randomized and counted by the same individual in a blind fashion in order to maintain consistency.

For immunohistochemistry, tissue samples fixed in 4% formalin in PBS were embedded in paraffin and cut into 2-5µm section. Tissue sections were rehydrated with Zylol to H_2O and blocked in 3% H_2O_2 in methanol for 15 min. Antigen retrieval was performed for 2 minutes in a pressure cooker in 0,01M Citrate Buffer pH6. Samples were cooled down in running tap H_2O and then blocked with Vector® blocker 1:5 for 20 minutes before rinsing with phosphate buffered saline with tween (PBST). Sections were incubated with anti-calbindin ([1#2136] Cell Signaling Technology, Inc., Danvers, MA, USA) (1:50) or anti-substance P ([240907] ABD serotech, Kidlington, UK) (1:100) for 60 minutes and then washed with PBST, followed by incubation with Vector®biotinylated Rabbit anti Rat (1:200) for 30 minutes, and washed with PBST. Tissue sections were incubated with Vector® ABC (Vectastain ABC Kit) 1:100 for 30 minutes and washed with PBST. Stained sections were developed with DAB Substrate for 10 minutes, washed with PBST and incubated with anti-CD3 ([A0452] Dako, Gauteng, South Africa) 1:100 for 60 minutes before washing with PBST. Sections were incubated with Rabbit Envision for 30 minutes and washed with PBST before developing them in Vector® VIP Substrate for 10 minutes. Sample sections were rinsed in distilled H_2O (dH_2O) and counterstained with Methyl Green for 5 minutes and rinsed in dH_2O. Sections were dehydrated with 96% alcohol, rehydatred in Zylol, and mounted with Entellen (Merck, South Africa). All histological sections were processed and stained by the department of surgery, Groote Schuur Hospital, Cape Town, South Africa. Digital images were captured using a Nikon 5.0 Mega Pixels Color Digital Camera (Digital SIGHT DS-SMc).

Intestine PAS/Glycoprotein assay

Jejunum pieces were isolated from mice and stored at -80°C in sterile PBS and later homogenised in 1ml PBS for 30 seconds at 4°C. Mucus glycoprotein production was determined by PAS assay following sample centrifugation at 3000rpm for 20 minutes at 4°C. Schiff reagent and 50% periodic acid solution (BDH Chemicals, Merck, Germany) were mixed with 7% ethanoic acid. Samples and standards dissolved in water were incubated for 2 hours at 37°C with 200µl of periodic acid solution. Following incubation, Schiff solution was added to glycoprotein solution and incubated at room temperature for 30 minutes for color development. Absorbance was read at 555nm (Mantle & Allen, 1978). A standard curve was done with each assay. All PAS assays were conducted by the department of surgery, Groote Schuur Hospital, Cape Town, South Africa.

Quantification of intestinal, hepatic and fecal *S. mansoni* egg burden

S. mansoni infected mice were euthanized by intraperitoneal injections of a combination of 0.3ml Anaket-V (Centaur Labs, RSA) and 2% Rompun (Bayer, Germany). Both the liver and intestine were rinsed in 4°C of 1.2% NaCl following dissection. Tissue samples were digested in 200ml of 5% potassium hydroxide (KOH) solution for 16 hours at 37°C. Digested samples and fecal matter suspended in isotonic solution overnight were disrupted by aspiration using a syringe and then filtered through a 150µm mesh sieve. Each sample from individual mice was counted in triplicate using an inverted microscope at 400X magnification. Values obtained were averaged and normalized according to the volume of the resuspension and fecal weight, and were presented as eggs per gram of tissue as previously described (Keiser et al., 2006; Kloetzel, 1967).

Detection of total and antigen-specific serum antibody by ELISA

ELISAs were performed as previously described (Mohrs et al., 1999). Blood samples were taken by tail vein bleeding and collected in plasma separator tubes (Microtainer™ SST, BD, USA). Samples were centrifuged for 10 minutes at 8000rpm and stored at -20°C. Analysis of antigen specific IgE and IgG2a were carried out by capture ELISA. Nunc Maxisorp 96-well plates (Nunc™, Denmark) were coated with either 10µg/ml of capturing antibody (Southern Biotechnology, US) or with 10µg/ml

of *S. mansoni* soluble egg antigen (SEA; BioGlab Limited, UK) at 4°C overnight in borate buffer (50mM), pH 9.6. Plates were washed and then blocked with 200µl of 2% milk powder overnight at 4°C. Samples in dilution buffer were added in serial dilutions and plates were incubated at 4°C overnight. Soluble egg antigen specific antibodies were detected by using alkaline phosphatase-conjugated goat anti-IgG2a (clone 017-YB09) (Southern Biotechnology, US). Total IgE (clone 84.1) was determined with monoclonal antibodies and detected with alkaline phosphatase-conjugated rat anti-IgE (clone 23G3). Total IgG2a was determined with monoclonal antibodies and detected with alkaline phosphatase-conjugated rat anti-IgG2a. Plates were washed and developed with 4-Nitrophenyl Phosphate Disodium Salt Hexahydrate (Fluka, BioChemika: Sigma-Aldrich, Austria) diluted in substrate buffer and measured at 405nm with 490nm set as reference on a Versamax microplate spectrophotometer (Molecular Devices, Germany). IgE was treated with streptavidin coupled to Horseradish Peroxidase (Boehringer Mannheim, Germany) for 1 hour at 37°C and measured using 3.3', 5.5'–tetramethylbenzidine (TMB) Microwell Peroxidase Substrate System [Lot# 070992 (substance A) and Lot# 071183 (substance B)] (MD, USA).

Physiological contractions

(see appendix for detailed methods and modification)

Cumulative method

The cumulative method was modified and performed as previously described (Tanovic et al., 2006). Segments of jejunum collected 10cm from the stomach pylorus or ileum collected 20cm from the stomach pylorus were placed in ice-cold Kreb's solution before being mounted vertically in organ bath chambers (Panlab, Spain) filled with 45ml of aerated (95:5% O_2/CO_2) Kreb's solution (NaCl 118.5 mM; KCl 4.74 mM; $CaCl_2$ 2.54 mM; KH_2PO_4 1.20 mM; $MgSO_4$ 1.19 mM; $NaHCO_3$ 25.0 mM; glucose 11.0 mM) maintained at 37°C. Tissue was fixed to a metal rod with a piece of thread from one end of the tissue attached to a transducer, and the other end to a metal rod at the bottom of a glass chamber. Tissue was subjected to a load of 9.9mN (1g) and incubated for 30 minutes, followed by stimulations with increasing concentrations of ACh every 30 seconds, without allowing tissue to go back to resting tension. Changes in isometric tension of the tissue along the longitudinal

axis were recorded with a tension-displacement transducer (ML T0201, PanLab, Spain). Data were analyzed using PowerLab^{TM} software (ADInstruments, Australia) and expressed as force (mN).

Single tissue/single concentration non-cumulative method

The cumulative method was modified and performed as previously described (Goldhill et al., 1997). 1cm long segments of the small intestine were collected into ice-cold Kreb's solution before being mounted vertically in organ bath chambers (Panlab, Spain) filled with 45ml of aerated (95:5% O_2/CO_2) Kreb's solution (NaCl 118.5 mM; KCl 4.74 mM; $CaCl_2$ 2.54 mM; KH_2PO_4 1.20 mM; $MgSO_4$ 1.19 mM; $NaHCO_3$ 25.0 mM; glucose 11.0 mM) maintained at 37°C. Seven pieces of intestine tissue were individually placed in organ chambers fixed to a metal rod with a piece of thread from one end of the tissue attached to a transducer, and the other end to a metal rod at the bottom of a glass chamber. Tissue pieces were subjected to a load of 9.9mN (1g) and incubated for 30 minutes, followed by each single piece of intestine being stimulated with a single concentration of ACh. Changes in isometric tension of the tissue along the longitudinal axis were recorded with a tension-displacement transducer (ML T0201, PanLab, Spain). Data were analyzed using PowerLab^{TM} software (ADInstruments, Australia) and expressed as force (mN).

Single tissue/various concentrations non-cumulative method

The cumulative method was modified and performed as previously described (Zhao et al., 2003). Segments of jejunum collected 10cm from the stomach pylorus or ileum collected 20cm from the stomach pylorus were mounted vertically in organ bath chambers (Panlab, Spain) filled with 45ml of aerated (95:5% O_2/CO_2) Kreb's solution (NaCl 118.5 mM; KCl 4.74 mM; $CaCl_2$ 2.54 mM; KH_2PO_4 1.20 mM; $MgSO_4$ 1.19 mM; $NaHCO_3$ 25.0 mM; glucose 11.0 mM) maintained at 37°C. Tissue was fixed to a metal rod with a piece of thread from one end of the tissue attached to a transducer, and the other end to a metal rod at the bottom of a glass chamber. Tissue was subjected to a load of 4.95mN (0.5g) and incubated for 30 minutes, followed by stimulations with 50mM KCl. Changes in isometric tension of the tissue along the longitudinal axis were recorded with a tension-displacement transducer (ML T0201, PanLab, Spain) as previously described (Unno et al., 2005). Following

tension displacement recordings, tissue was allowed to go back to resting tension (4.95g) and washed with fresh Kreb's solution for 10 minutes before being subjected to varying concentrations of ACh ranging from -9 to -3 LOG [M], with 10 minutes washes with fresh Kreb's solution between each concentration. Changes in isometric tension were recorded following each concentration challenge, and tissue allowed to return to resting tension following each tension displacement recording. Data were analyzed using PowerLab™ software (ADInstruments, Australia) and expressed as force (mN) or as force normalized by individual tissue weight (mN/mg).

Statistical analyses

Values are represented as mean ± SEM with significant differences determined by ANOVA and Tukey's post-hoc tests performed if $p<0.05$, or unpaired t-test. Before data from individual experiments were pooled, ANOVA analyses were conducted for each treatment group e.g. non-infected groups: experiment 1 vs. experiment 2. In cases of no significant differences detected between experiments, they were pooled and re-analyzed by ANOVA. Concentration point-to-point ANOVA analyses were conducted for contractions experiments. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ (GraphpadPrism™, San Diego, California, USA).

Optimization of Isolated Intestine Tissue Contraction Method

(Part 2)

TCO.2.0 Introduction

The GI tract experiences continuous exposure to pathogens (Collins, 1996). Depending on the type of luminal factors, the immune system is able to distinguish between harmful antigens and useful substances (Mowat, 1997), hence triggering an appropriate response. Upon *N. brasiliensis* infection, the host develops integrated responses that incorporate smooth muscle functionality (Collins, 1996), the enteric nervous system (Sharkey, 1996), as well as the enteric immune system (Zhao et al., 2003). In cases of nematode infection, the functionality of the GI tract is altered by enhanced mucus production in the lumen (Miller et al., 1981), as well as an increase in intestinal propulsive activity (Alizadeh, 1987). Both of these activities are hypothesized to aid in parasite expulsion as the mucus drains the parasite inhibiting its mobility, and the luminal villi in turn "sweep" the parasite in transit to be expelled from the host. This motility change is associated with smooth muscle hypercontractility (Farmer, 1981). Infected murine models demonstrate intestinal inflammation that is characterized by altered GI motility. This alteration in smooth muscle is capable of inducing both functional and structural changes in the smooth muscle cells that may influence smooth muscle contractile responses.

Following *S. mansoni* infection, T_H1-type responses are activated during the initial stages of infective cercariae in murine experimental models (Pearce & MacDonald, 2002). Adult worms later produce eggs at 5 weeks, and a T_H2-type response is then activated against egg antigens (Herbert, 2004). During the chronic phase of infection that is usually exhibited at 8 weeks p.i., eggs gather in the liver (Taylor et al., 2009), and mice that do not express IL-4Rα die from inflammatory reactions to the parasite (Leeto et al., 2006). Generally, host protection against *S. mansoni* infection rests on the host's ability to successfully signal IL-4 and IL-13 via IL-4Rα. Mice expressing IL-4 have been shown to demonstrate smooth muscle hypercontractility (Vallance et al., 2007) when stimulated with carbachol compared to their non-infected controls, suggesting the significance of IL-4 in neurotransmitter driven hypercontractility of the smooth muscle. While IL-4 treated mice demonstrated increased contractility in response to ACh stimulation, IL-13 treated mice on the other hand demonstrated no significant differences to non-infected controls in ACh driven smooth muscle hypercontractility (Zhao et al., 2003). STAT-6$^{-/-}$ mice demonstrated attenuated smooth muscle hypercontractility 9 days p.i. with *N.*

brasiliensis in response to ACh stimulation compared to wild-type controls (Zhao et al., 2003).

ACh has been reported to be the primary neural regulator of GI motility, with ability to directly influence intestinal smooth muscle contractility (Burks, 1994). During nematode infection, this ability may be altered, either due to direct effects on the cholinergic system of the GI tract, or via indirect influences of the immune products affecting cholinergic responsiveness. In light of the possible alteration that may be induced on smooth muscle cells by *N. brasiliensis* and *S. mansoni* infection, KCl was used as a stimulus for testing any functional changes that may have occurred as KCl is capable of inducing contractile responses independent of receptor-ligand interaction (Unno et al., 2005). Both carbachol and ACh have been shown to signal via the M_3 receptor, and it has been reported that in the absence of this receptor contractile responses to these muscarinic agonists is either attenuated or abrogated (Matsui et al., 2002). Horsnell et al., 2007 reported a positive correlation between M_3 receptor mRNA expression and IL-4Rα expression. In this study they demonstrated that in the absence of IL-4Rα there is a reduction in M_3 receptor mRNA expression, suggesting that there may be a bidirectional communication network between the enteric immune system and the enteric nervous system. These associations are explored, along with the possible mechanisms that may be driving the interaction.

The aim of this project was to determine the role of IL-4Rα expression on smooth muscle cells in cholinergic induced contractile responses following both *N. brasiliensis* and *S. mansoni* infections, with concentration-responses referred to as tension (Vallance et al., 2007; Zhao, 2005). The goal was to investigate if immune products have an effect on cholinergic products' influence on smooth muscle cells contractility, and if the effects are *N brasiliensis* and/or *S. mansoni* infection dependent.

In order to investigate isolated intestine tissue *ex vivo* for contractile responses, a tissue-organ bath system was employed, enabling controlled manipulation of oxygen, temperature, tissue tension, as well as stimulus administration. The ability of isolated intestine tissue to contract *ex vivo* depends on the quality of buffer the tissue is placed in, i.e. pH, temperature, as well as chemical compounds making up the buffer. The load to which tissue is stretched during the experiments is of great

importance in that it determines the length of the tissue. Smooth muscle contraction results when the active filaments attach (they are not attached in a resting state), leading to the rotation of myosin heads which in turn "push" the actin filaments, thereby shortening (contracting) the sarcomere causing both movement and tension. When the sarcomere is stretched to a load leading to a length that causes the myosin and actin filaments to "fail" to overlap (overstretched), no active tension will develop (Ghez, 1991). Isometric contractile tension is produced when tissue muscle length is fixed e.g. to a specific load, and this tension of contraction dependents on the rate of myosin and actin filaments movement.

Organ bath apparatus

Isolated tissue preparations require control of temperature which is supported by the fact that the organ bath is water-jacketed for the purpose of providing stable temperature that may be adjusted. The water bath is fitted with coils that are connected to a thermostat regulating water temperature. Also found in the water bath are individual organ chambers for accommodating tissue to be manipulated. These organ chambers receive constant supply of gas that bubbles through the buffer used in experiments via glass frits found at the bottom of individual organ chambers. The amount of gas supplied to the tissue may be controlled. A metal rod placed in an organ chamber is used for attaching one end of a piece of isolated tissue by thread to an isometric transducer—that measures changes in tension induced by tissue following any stimulation—while the other end of the tissue is hooked to the bottom of the rod. All individual isometric transducers are connected to amplifiers that enhance induced tension responses and are translated via a PowerLab™ that is used for recording and analyzing responses [Figure 2.0].

Experiment overview

Wild-type mice infected with *N. brasiliensis* have been shown to clear infection in less than two-weeks and demonstrated peak worm burdens at day 7 p.i. (Barner et al., 1998). The ability of the host to clear infection rests on successful generation of a T helper 2 (T_H2)-type response (Michels et al., 2006) as well as responses to the T_H2 cytokines IL-4 and IL-13 (Loke et al., 2007; McKenzie et al., 1998). Both cytokines signal through the IL-4Rα (Brombacher, 2000), and initiate STAT-6-dependant mechanistic responses to resolve infection. A T_H2 response is

characterized by heightened expression of immunoglobulins such as IgG1 and IgE (Barner et al., 1998; Fallon et al., 2002). Activated effector cells migrate to the site of invasion for host protective inflammatory responses (Fallon et al., 2002). IL-4 and IL-13 that are produced during infection have been reported to lead to increased smooth muscle cell contractility (Zhao et al., 2003) as tissue treated with these cytokines demonstrated hypercontractile responses to carbachol stimulation. Immune responses from immuno-competent wild-type mice result in intestine tissue inflammation (Akiho et al., 2002), secretion of large amounts of mucus following goblet cells hyperplasia (Miller et al., 1981), as well as heightened enteric smooth muscle contractions (Zhao et al., 2003).

In order to establish the contraction method, isolated tissue was stimulated with both KCl and ACh. KCl is a more general stimulus in the sense that it is capable of bypassing G-protein-coupled receptors (GPCR) hence activating smooth muscle cells via voltage-operated Ca^{2+} channels, leading to contraction (Ratz, 2005). Both potassium and chloride ions enter smooth muscle cells via leak channels that enable them to bypass GPCRs. KCl is therefore used in these experiments as a "simple" stimulus (Ratz, 2005) to evaluate tissue's ability to contract independent of a specific receptor-ligand interaction—in this case ACh. Before exposure to KCl, tissue was weighed as a variable parameter to normalize contractile results by dividing the acquired tension with the respective weight following ACh stimulation. Intestine tissue contractile responses to ACh stimulation were compared between BALB/c mice and IL-4Rα$^{-/lox}$ mice at day 7 following *N. brasiliensis* infection in order to determine if hemizygosity influences smooth muscle contraction.

N.B. Due to the limited number of chambers in the organ-bath and the time constraints during experimentation, a small sample size was used, and data pooled where stated.

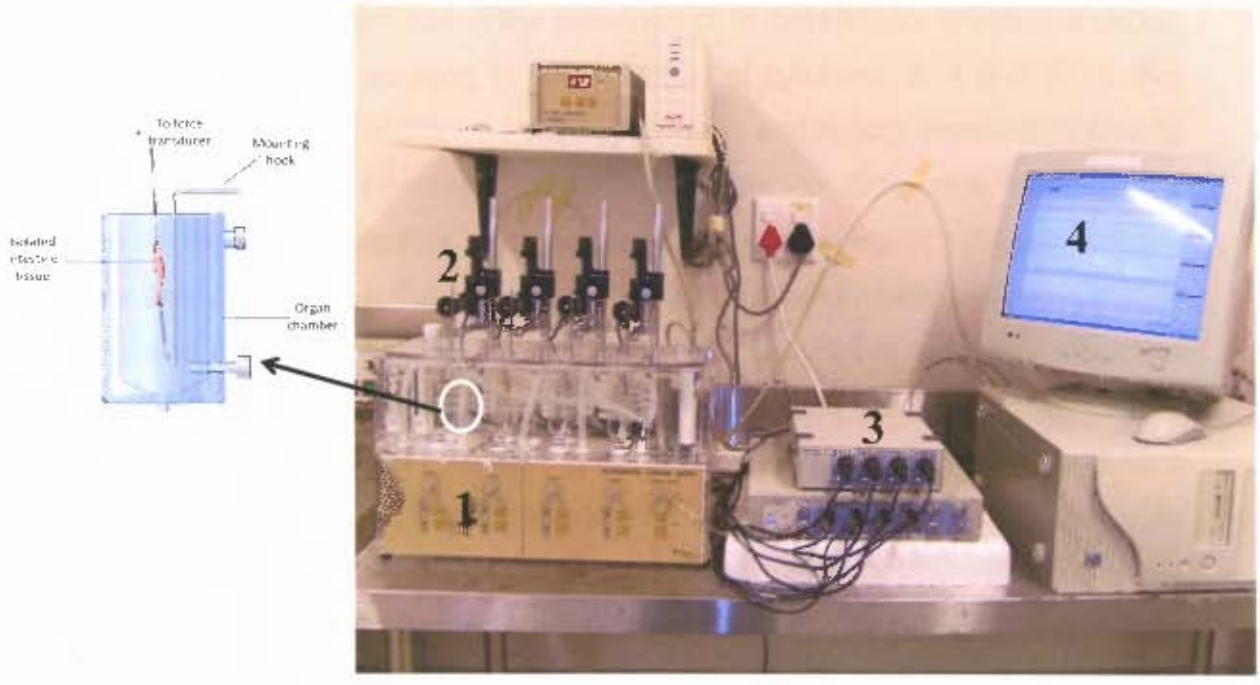


Figure 2.0

Representation of water-jacketed organ bath (Panlab, Spain) **(1)** with transducer **(2)** and PowerLab™ system (ADInstruments, Australia) **(3)** which translates the signal to measure tissue isometric tensions to the computer **(4)**. (Picture taken by T. Brombacher, 2008).

TCO.2.2 Results

Nippostrongylus brasiliensis *infection in BALB/c mice*

Previous studies have shown that in the presence of *N. brasiliensis* infection a strong T_H2 response is induced, resulting in the induction of cytokines IL-4 and IL-13 that have been shown to influence goblet cell hyperplasia and mucus production among infected tissue (Levy & Frondoza, 1983). In order to determine if infections conducted in these experiments were successful, *N. brasiliensis* infected BALB/c mice were analyzed at day 7 or day 10 p.i. for goblet cell hyperplasia and mucus production and compared to non-infected controls. Qualitative analyses of goblet cell hyperplasia were identified by an increase in red stains within the cells [Figure 2.1.A], and this was quantified by counting the number of enlarged mucus-producing goblet cells [Figure 2.1.B], as well as the amount of mucus produced by goblet cells measured from tissue homogenates [Figure 2.1.C] revealing goblet cell hyperplasia as well as increased mucus production following infection. Together, these results confirm that *N. brasiliensis* infection induced a T_H2 response characterized by goblet cell hyperplasia and heightened mucus production, accompanied by the absence of worms on intestine tissue at day 10 p.i. [Figure 2.1.1A].

Immuno-competent mice are capable of triggering a T_H2-type response that leads to elevated expressions of IgE (Barner et al., 1998; Fallon et al., 2002). In order to determine if the same was characteristic of BALB/c mice used in this experiment, blood samples were drawn from infected mice at day 7 and day 10 p.i. Antibodies IgE and Ig2a levels were determined from blood plasma by sandwich ELISA. Measurement of total antibody responses showed significantly enhanced levels of IgE at day 10 p.i. compared to both non-infected controls and day 7 p.i. [Figure 2.1.1B]. In contrast, no differences were observed between infected and non-infected blood plasma IgG2a levels [Figure 2.1.1C]. These results confirmed that *N. brasiliensis* enhances a T_H2 response characterized by increases in IgE production (Chen et al., 1995), and that IgG2a is not required for *N. brasiliensis* expulsion (Urban et al., 1998). Together, these results confirmed successful infection and protective T_H2-driven immune responses in BALB/c mice.

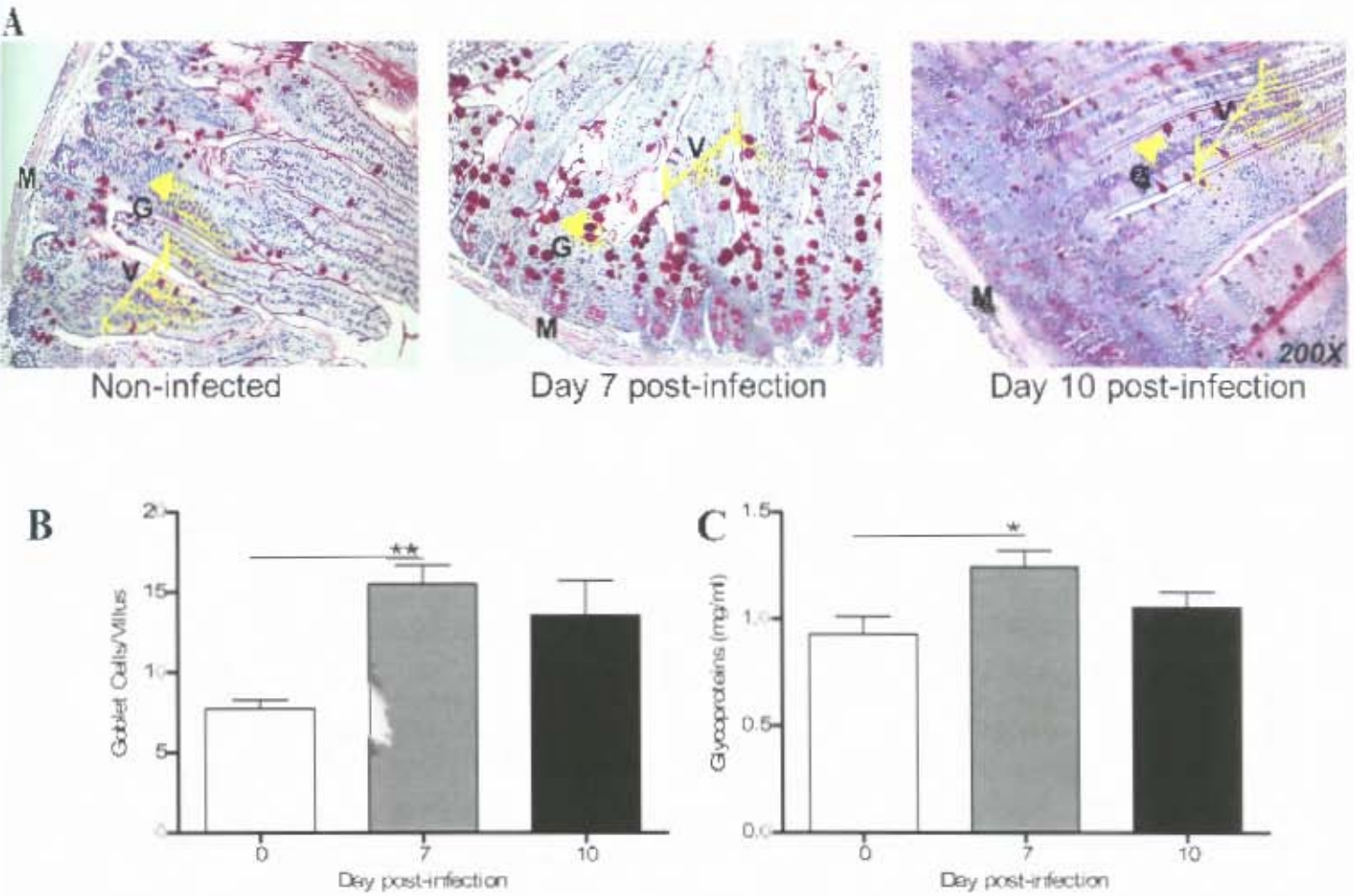


Figure 2.1

Effects of N. brasiliensis *infection on T_H2-type responsiveness in BALB/c mice*

BALB/c non-infected, day 7 and day 10 p.i. (n=8-12) mice jejunum exposed to *N. brasiliensis* were investigated for classical markers of T_H2-type responsiveness. **(A)** Periodic acid Schiff's staining of tissue cross-sections was conducted to investigate any possible alterations following infection (200X magnification; M: Muscularis, G: Goblet cells [shown by arrow heads], V: Villus [shown by bordered line]). **(B)** Tissue's goblet cell results are expressed as number of goblet cells per villus, where the numbers of cells in five villi were measured from 1 tissue section per individual mouse and divided to give a relative representation per villus with n=4 and repeated over 3 experiments. **(C)** Mucus glycoprotein production was determined from individual jejunum tissue homogenates by periodic acid Schiff's assay and expressed in mg/ml. Results are shown as mean±SEM, ANOVA * $p<0.05$; ** $p<0.01$, and are pooled from 3 different experiments.

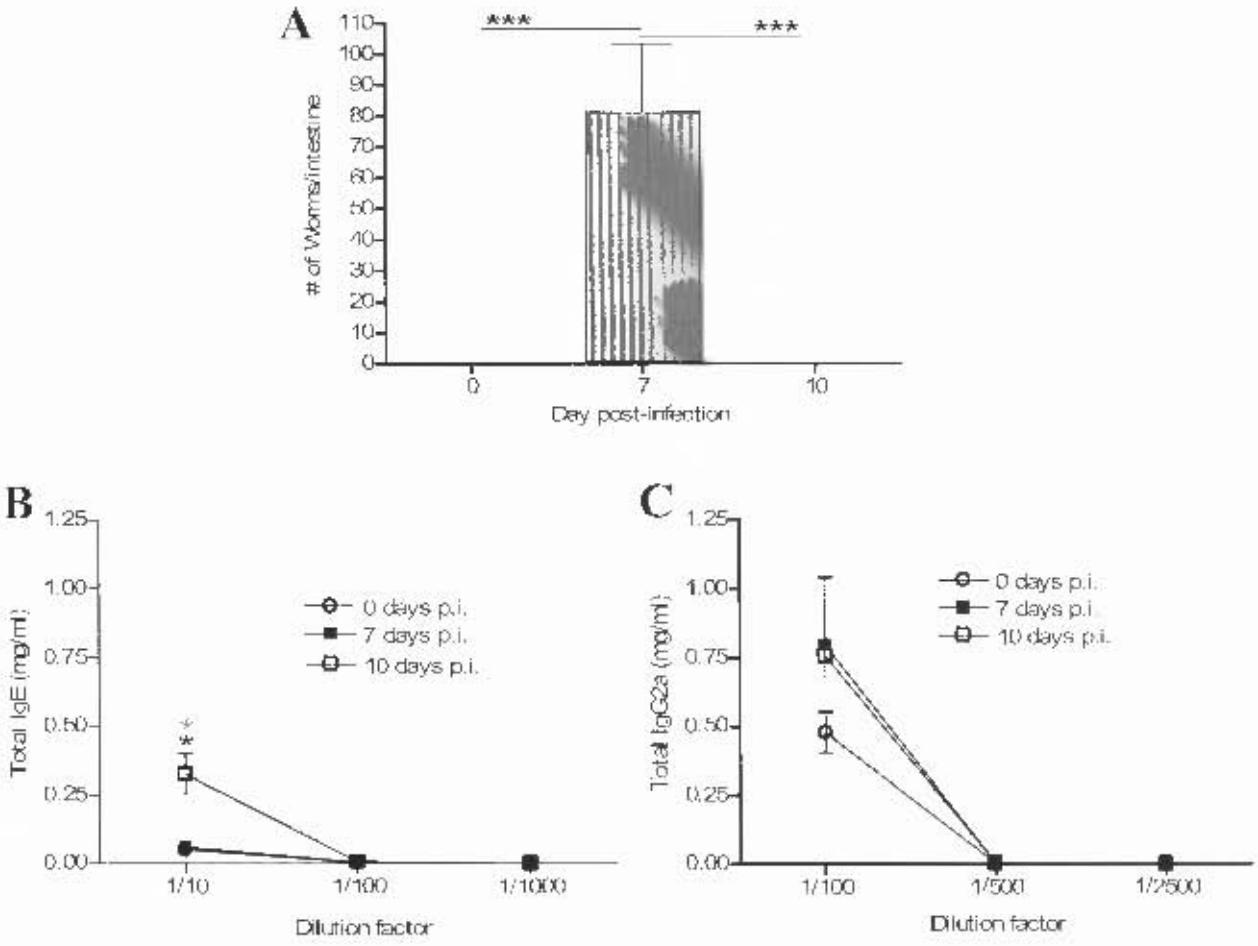


Figure 2.1.1

Effects of N. brasiliensis *infection on T_H2-type responsiveness in BALB/c mice*

BALB/c non-infected, day 7 and day 10 p.i. (n=8-12) mice jejunum exposed to *N. brasiliensis* were investigated for classical markers of T_H2-type responsiveness. **(A)** Intestinal worm expulsion following *N. brasiliensis* infection was carried out by counting adult worms from each mouse whole intestine tissue with n=4 and repeated over 2 experiments. Blood serum antibody expression following *N. brasiliensis* infection was studied after drawing blood from each mouse n=4 over 2 experiments, and serum concentrations of total IgE **(B)** and total IgG2a **(C)** were determined. Results are shown as mean±SEM, ANOVA * $p<0.05$; *** $p<0.001$, and are a representation of 2 independent experiments.

Optimization of intestine tissue contraction methods

This work describes the optimization of isolated intestine tissue contractility protocols. It incorporates comparing obtained results to those previously reported by other laboratories. Three different physiological intestine tension measuring techniques were investigated, namely, the cumulative, the single tissue/single concentration non-cumulative method, as well as the single tissue/various concentrations non-cumulative method, based on published literature. *N. brasiliensis* studies tend to use the jejunum part of the small intestine in their whole-tissue contraction experiments (Zhao et al., 2003; Zhao et al., 2006), while *S. mansoni* studies use the ileum (De Man et al., 2002) as these are areas of the intestine that are mostly affected. In order to eliminate any possible differences in tissue contractile responses that may be due to the area of the small intestine used, the jejunum and ileum were chosen as areas to use in all consecutive experiments. This was done to ease comparisons between experiments conducted in this study to those of other publishers. It has been noted that the tissue's tension of contraction depends on the length of the muscle in question among other factors (Ghez, 1991). Based on this information all pieces of tissue used in the project were 1cm long and loaded to a specific tension for measuring isometric contractile tensions.

Establishing the cumulative method

The cumulative method for studying isolated tissue contractile responses was adapted from a study conducted by Tanovic et al., 2006 who infected rats with *Trichinella spiralis* and investigated mechanical responses of each jejunum piece of tissue to varying concentrations of ACh, preloaded with 9.9mN. Tissue pieces were equilibrated in Krebs buffer for 1 hour and the amplitude of contractile responses was recorded. The method was modified in this project by equilibrating tissue in Krebs buffer for 30 minutes as this length of time appeared suitable because at this time-point baseline contractile responses were stabilized. IL4R$\alpha^{-/lox}$ mice on a BALB/c background inoculated with *N. brasiliensis* L3 studied at day 0 (non-infected) p.i., day 7 p.i. and day 10 p.i. were used in this experiment to investigate whether the cumulative method for studying isolated tissue contractile responses to ACh was suitable for yielding reliable concentration-response curves. The jejunum part of the small intestine measured 10cm from the stomach pylorus was isolated, and 1cm

long pieces were kept in ice-cold Krebs buffer until contractile experimentation. Each individual tissue piece was stretched to a load of 9.9mN, equilibrated for 30 minutes in oxygenated Krebs buffer at 37°C, and stimulated with varying concentrations of ACh ranging from -9 LOG [M] to -3 LOG [M] every 30 seconds. Tissue was not allowed to go back to resting state between stimulations. Despite enhanced contractile tension as observed on the PowerLab™ data traces (not shown) in response to ACh stimulation, drastic tissue tension loss was evident, resulting in negative concentration-response curve units that resembled a lack of tissue response to ACh stimulation among naive mice and minimal concentration-dependent responses at day 7 p.i. IL4Rα$^{+/lox}$ mice at day 10 p.i. demonstrated significant contractile ability against both non-infected and day 7 p.i. groups [Figure 2.2A]. The cumulative method revealed its shortfalls by exhibiting negative concentration-response curves resulting from rapid tissue tension loss enhanced by consecutive stimulations with ACh every 30 seconds. This result demonstrated that tissue filaments may require some "rest" between stimulations in order to achieve their maximum contractile ability per concentration.

Establishing the non-cumulative method

Single tissue/single concentration:

The non-cumulative method for studying isolated tissue contractile responses was adapted from a study conducted by Goldhill et al., 1997 who treated BALB/c mice with IL-4 and isolated the entire small intestine, using only 5-10cm segments in the middle of it for contractile experiments. Each piece of tissue was equilibrated for 20 minutes in Krebs buffer, and stimulated with varying concentrations of ACh. Tissue was stretched to a load of 9.9mN, and buffer replaced every 10 minutes throughout the study. Non-cumulative concentration-response curves were constructed in the same tissue. The method was modified in this project by isolating the entire small intestine following *N. brasiliensis* infection at day 7 or day 10 p.i. In order to minimize tissue handling and the possibility of altering contractile ability, each 1cm long piece of tissue was stimulated with a different concentration of ACh after being kept in cold Kreb's buffer until contractile experimentation. This eliminated the need to replace buffer every 10 minutes. This method was adapted for the purpose of determining if it was suitable for studying maximal enhanced tissue contractile

responses. In this study, each individual piece of intestine tissue was stretched to a load of 9.9mN, equilibrated for 30 minutes in oxygenated Krebs buffer at 37°C, and stimulated with a different individual concentration of ACh. This serves to say that tissue in chamber number 1 was stimulated with -9 LOG [M] and the one in chamber number 7 with -3 LOG [M]. Enhanced contractile responses to ACh are seen at day 7 p.i. compared to their non-infected controls, however, variability was observed in these concentration-response curves [Figure 2.2B]. These results reveal that this non-cumulative method for studying physiological contractions does not demonstrate "ideal" concentration-response curves and therefore not suitable for studying mouse strains in our laboratory. The variability in concentration-response curves observed while using this non-cumulative method to study physiological contractile responses to ACh suggests that the possible uniqueness of each piece of tissue may influence its ability to respond to a stimulus, and therefore attempting to acquire a concentration-response curve from such a setup proved to be limiting.

Single tissue/various concentrations:

The single tissue/various concentrations non-cumulative (*sv*.non-cumulative) method for studying isolated contractile responses was adapted from a study conducted by Zhao et al., 2003 who infected BALB/c mice with *N. brasiliensis* and stimulated segments of the jejunum with ACh. As soon as a piece of tissue reached its maximum contractile response, it was washed, and the next ACh concentration was added at 10 minutes intervals. Tissue pieces were equilibrated in Krebs buffer for 30-45 minutes, and stretched to a load of 9.9mN. This method was modified in this project by stretching tissue to a load of 4.95mN because 9.9mN appeared to be overstretching tissue, demonstrated by rapid tension loss, thus inhibiting maximal tissue contractile ability in response to stimulation. Krebs buffer was replaced after each tissue reached its maximum contractile response. Tissue was allowed to go back to its initial "resting" tension—4.95mN—for 10 minutes in fresh buffer pre-warmed to 37°C between ACh stimulations of varying concentration. BALB/c mice inoculated with *N. brasiliensis* L3 and studied at day 7 and day 10 p.i. along with non-infected controls were used in this experiment to investigate whether the *sv*.non-cumulative method for studying isolated tissue contractile responses to ACh was suitable for yielding reliable concentration-response curves. The jejunum part of the small intestine measured 10cm from the stomach pylorus was isolated, and 1cm

long pieces were placed in oxygenated Krebs buffer immediately. Each individual tissue piece was stretched to a load of 4.95mN, equilibrated for 30 minutes in oxygenated Krebs buffer at 37°C before being stimulated with 50mM KCl, followed by stimulation with varying concentrations of ACh ranging from -9 LOG [M] to -3 LOG [M]. Krebs buffer was changed, and tissue washed for 10 minutes between stimulations. Enhanced contractile tension was observed in response to ACh stimulation with positive concentration-response curves represented in all treatment conditions [Figure 2.2C]. This demonstrated that the *sv.*non-cumulative method for studying physiological contractions is suitable for use. These results suggest that the ideal method for acquiring optimal contractile responses to ACh stimulation is the *sv.*non-cumulative method as it allows tissue to go back to its resting state, hence giving the muscle a chance to re-align its filaments, getting them ready to overlap and develop shortening of the muscle known as contraction upon stimulation (Ghez, 1991).

Suitable tissue stretch load for attaining optimal length suitable for contractile enhancement has been identified as 4.95mN in these experiments as 9.9mN adapted by other laboratories (Goldhill et al., 1997) appeared to lead to an overstretch of tissue that possibly attenuated the myosin filaments' ability to contact actin filaments, thus affecting muscle shortening in the presence of stimuli. The 9.9mN stretch load was able to yield contractile responses to ACh; however, 4.95mN appeared to yield better enhanced contractile responses and was chosen as the stretch load for all future experiments. Enhanced intestine tissue tension at day 7 p.i. when using both non-cumulative methods is not observed when the cumulative method is employed due to rapid intestine tissue tension loss observed in the cumulative method.

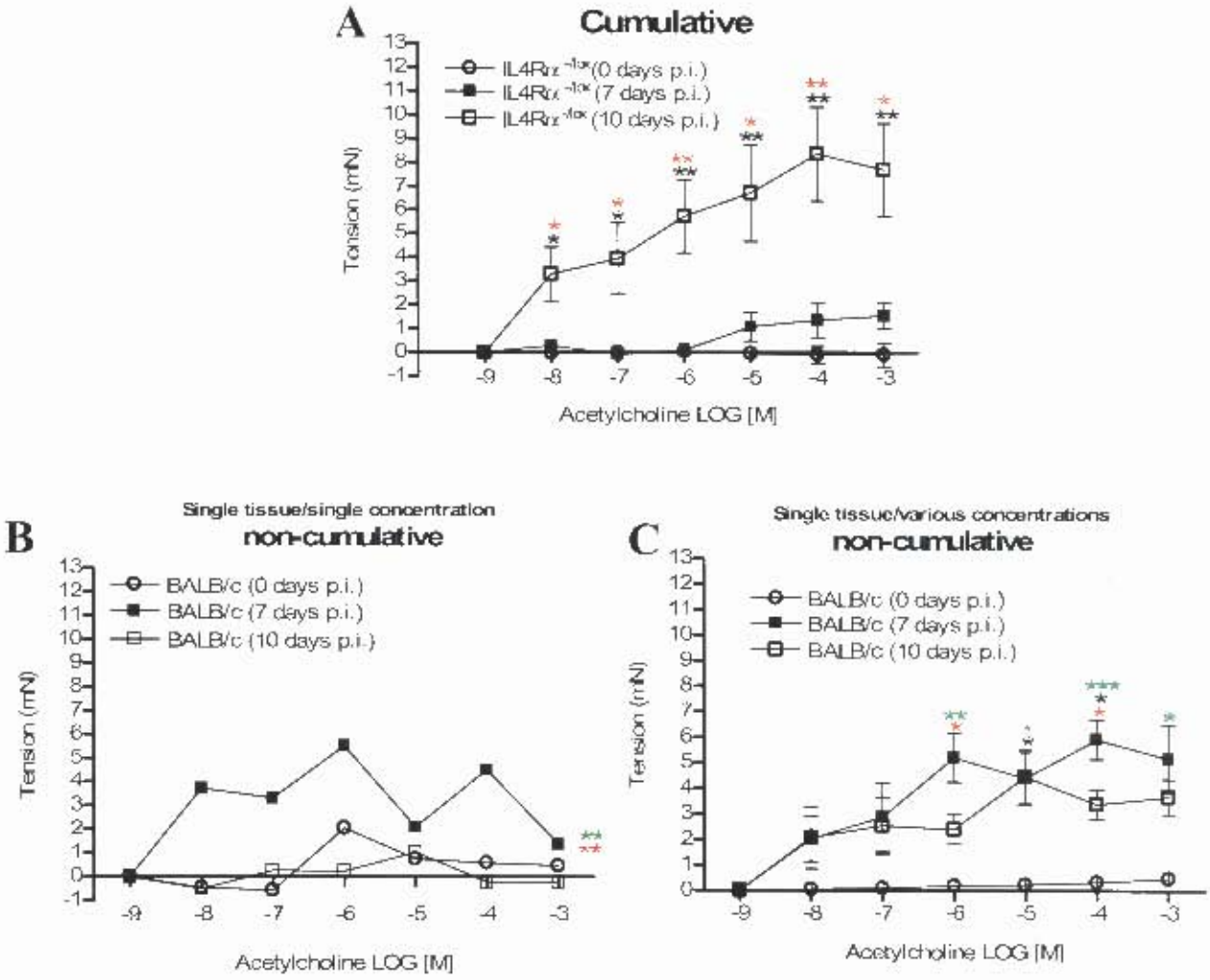


Figure 2.2

Effects of various methods for studying isolated tissue contractile responses to acetylcholine

(A) Cumulative method: IL-4Rα$^{-/lox}$ mice of BALB/c background non-infected, day 7 p.i., and day 10 p.i. (n=4) mice jejunum exposed to *N. brasiliensis* were stimulated with varying concentrations of ACh per tissue, and concentration dependent responses were investigated. **(B)** Non-cumulative method: BALB/c non-infected, day 7 p.i. and day 10 p.i. (n=4) mice whole intestine exposed to *N. brasiliensis* were stimulated with varying concentrations of ACh per piece, and concentration-dependent responses were investigated. **(C)** Non-cumulative method: BALB/c non-infected, day 7 p.i., and day 10 p.i. (n=4) mice jejunum exposed to *N. brasiliensis* were stimulated with ACh. [Non-infected vs. day 7 p.i.; Non-infected vs. day 10 p.i.; day 7 p.i. vs. day 10 p.i.]. Figure is a representation of 2 independent experiments. Results are shown as mean±SEM, ANOVA for individual concentration points * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

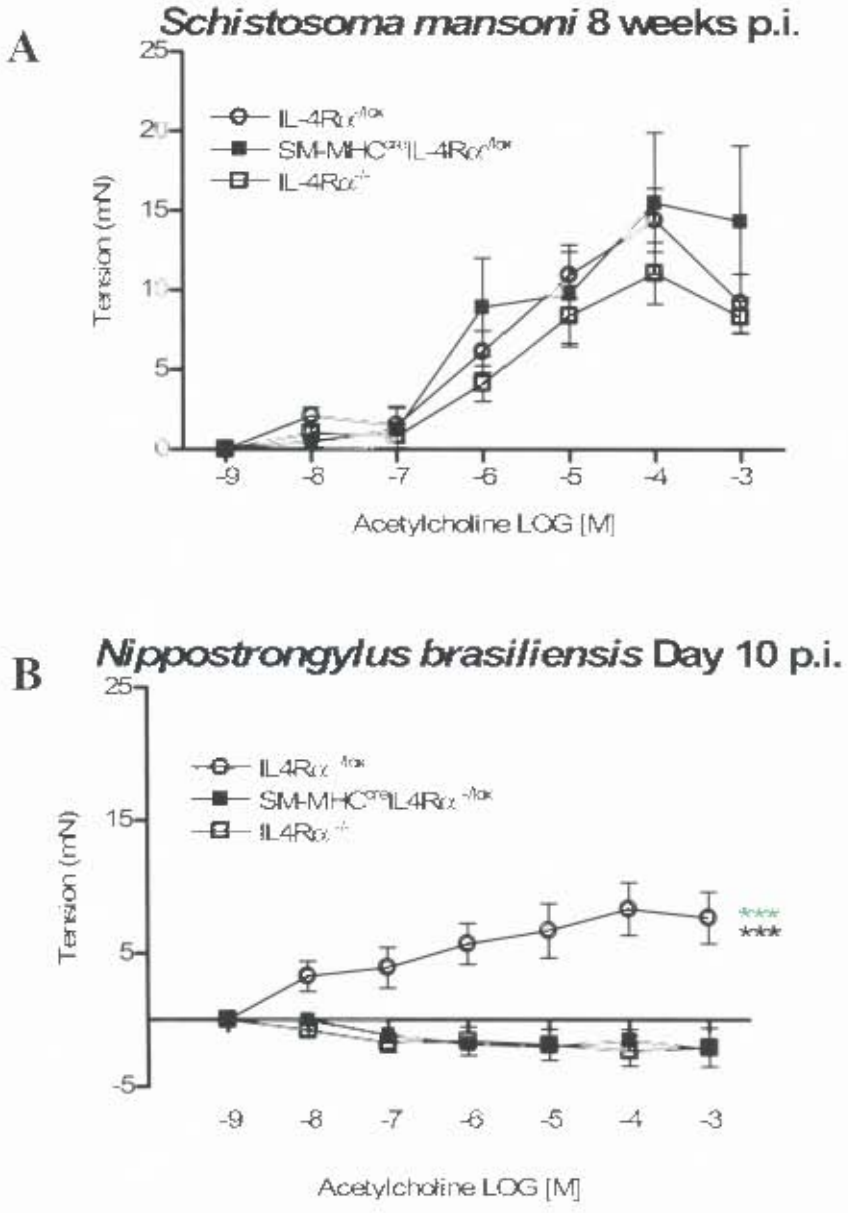


Figure 2.3

Effects of cumulative method on IL-4Rα-deficient mice following S. mansoni and N. brasiliensis infection

(A) IL-4Rα-deficient mice (IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα ^{lox}, and IL-4Rα$^{-/-}$) of BALB/c background inoculated with 100 cercariae studied at p.i. 8 weeks (n=4), and **(B)** those infected with *N. brasiliensis* studied at day 10 p.i. (n=4) were stimulated with varying concentrations of ACh (-9 to -3 LOG [M]) per tissue, and concentration dependent responses were investigated. These are a representation of 2 independent experiments. Results are shown as mean±SEM, ANOVA for individual concentration points * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Contraction studies in Nippostrongylus brasiliensis *infected BALB/c mice*

BALB/c mice inoculated with ~750 *N. brasiliensis* L3 s.c. and killed at day 7 or day 10 p.i. had the jejunum isolated and analyzed for isometric contractile responses using the *sv.*non-cumulative method. This was for the purpose of determining the validity of the *sv.*non-cumulative method. At the beginning of each experiment, 1cm pieces of tissue were weighed on an analytical balance before conducting physiological contractions experimentation. During physiological contractility studies, tissue was initially stimulated with 50mM KCl and then washed for 10 minutes following maximum amplitude readings. The same pieces of tissue were then stimulated with varying concentrations of ACh. Data gathered from this experiment included individual tissue weight, tension expressed in mN (Mills et al., 2008) for KCl and ACh enhanced tension, tension normalized to tissue weight expressed in mN/mg (Won et al., 2002) for ACh enhanced tension.

Tissue weights at day 7 and day 10 p.i. were higher than those of non-infected controls, with statistical significance between day 10 p.i. and non-infected controls [Figure 2.4A]. Tension increases in response to KCl were found at day 7 and day 10 p.i., with statistical significance compared to non-infected controls [Figure 2.4B]. Tissue from infected mice showed strikingly increased ACh concentration-dependent tension compared to non-infected controls [Figure 2.4C-D]; however, the power of significance was lower when the tissue weight was considered (mN/mg) [Figure 2.4D]. Together, these results show that *N. brasiliensis* infection leads to an increase in jejunum tissue weight which may be due to inflammation, as well as an increase in contractile responsiveness to KCl or ACh due to possible tissue structural alteration. It is therefore concluded that *sv.*non-cumulative method is the most suitable for studying tissue contractile responses following *N. brasiliensis* infection, and that infection leads to increased tissue weight, as well as increased contractility in response to stimulation.

For the investigation of the role of IL-4Rα-responsiveness on smooth muscle cells specific IL-4Rα deficient mice (SM-MHC^{cre}IL4Rα$^{-/lox}$), hemizygous IL4Rα$^{-/lox}$ control littermate mice would be the better control than wild type BALB/c mice, as previously used in *N. brasiliensis* infection studies (Horsnell et al., 2007). Therefore, ACh-induced contractility at day 7 p.i. between IL4Rα$^{-/lox}$ and BALB/c mice strains was

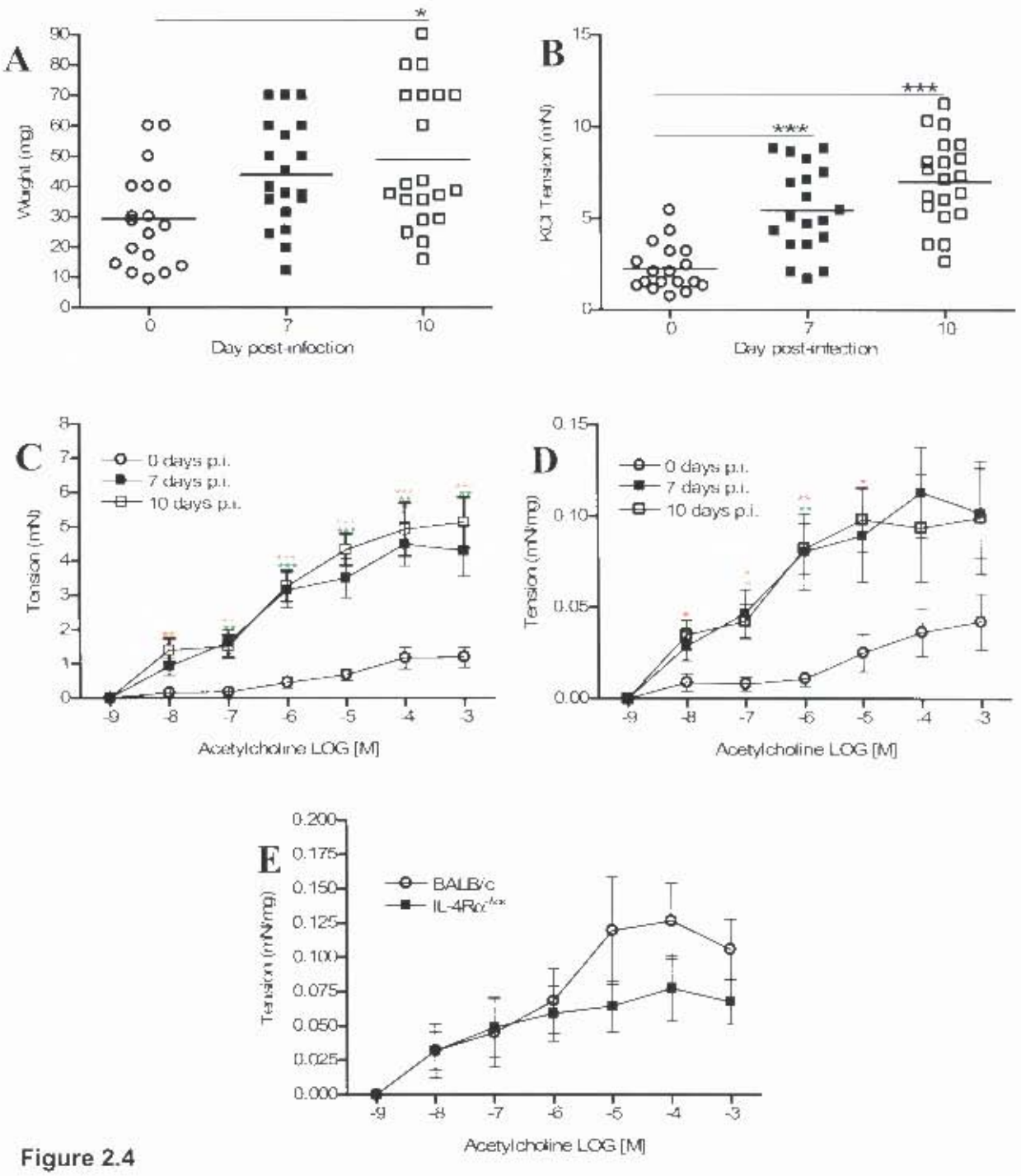


Figure 2.4

The role of N. brasiliensis *infection on isolated intestine tissue contractile responses*

BALB/c non-infected (n=18), day 7 p.i. (n=19), and day 10 p.i. (n=20) mice 1cm long isolated jejunum pieces were compared for **(A)** tissue weight, **(B)** contractile responses to 50mM KCl, contractile responses to ACh presented as either **(C)** force or **(D)** as force divided by tissue weight. [Non-infected vs. day 7 p.i.; Non-infected vs. day 10 p.i.]. Results are shown as mean±SEM, ANOVA * $p<0.05$; ** $p<0.01$; *** $p<0.001$, and pooled from 5 different experiments. **(E)** BALB/c and IL4Rα$^{-/-}$ mice (n=4) studied at day 7 p.i. were stimulated with ACh and concentration-dependent responses were determined. Tissue's contractile results are shown as mean±SEM, ANOVA or t-test for individual dose points * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Figure is a representation of 3 independent experiments.

Discussion

The objective of this project was to understand neuro-immune interactions affecting intestine smooth muscle contractility and host resistance to helminth infection. For this purpose, a method for measuring isometric tension on isolated intestine tissue had to be established in the laboratory.

From extensive literature search, the two commonly used methods for studying isometric tension on isolated intestine tissue are the cumulative and non-cumulative methods. Both methods were tested using a tissue-organ bath system which enabled the control of oxygen, temperature, tissue tension, as well as stimulus administration.

The cumulative method is based on a single piece of intestine tissue which is stimulated with varying concentrations of ACh within short intervals, without letting tissue go back to resting tension between stimulation (Tanovic et al., 2006). When tested in the laboratory, the method resulted in drastic tissue tension loss and negative concentration-response curve units among non-infected mice, even though this method was reported to have worked well in previous literature. Minimal concentration-dependent responses were observed at day 7 p.i. with *N. brasiliensis*, indicating tissue exhaustion. The lack of success in using this method could be attributed to the different species used. Tanovic et al., 2006 evaluated possible intestine motor alterations during inflammation with *Trichinella spiralis* in rats, using infected jejunum tissue and non-infected ileum tissue. Interestingly, this method yielded excessive tension loss in *N. brasiliensis* infected mouse tissue, but resulted in an ACh concentration-response curve from *S. mansoni* infected mouse tissue. However, due to high variability, ACh-induced tension differences were not observed between IL-4Rα-deficient mouse strains. Possible reasons include the different intestinal tissues used (jejunum for *N. brasiliensis* studies and ileum for *S. mansoni* studies) and the different pathology caused by the two helminths. While *N. brasiliensis* are in the lumen and cause local inflammation, the eggs of *S. mansoni* transit through the intestinal tissue into the lumen, and many get stuck in the muscularis causing drastic inflammation and granulomas in the tissue.

The non-cumulative method can be achieved by either stimulating independent pieces of isolated intestine tissue with single ACh concentrations (single piece/single

concentration), or by stimulating one piece of intestine tissue with increasing concentrations of ACh paired with resting/washing intervals between each concentration (Goldhill et al., 1997; Zhao et al., 2003), which is called single piece/various concentrations non-cumulative (*sv*.non-cumulative) method in this study. Using a single piece/single concentration method to construct a concentration-response curve yielded a high degree of variation with negative concentration-response curve units and no clear ACh-induced tension, particularly during infection. This method was adapted and altered to eliminate tissue washing steps in order to minimize tissue handling time, but reliable concentration-response curves were not achieved during infection studies.

The *sv*.non-cumulative method using one piece of tissue and sufficient washing/resting time, stretched to a load of 9.9mN (Goldhill et al., 1997; Zhao et al., 2003) was, after testing, adapted to 4.95mN as previously published (De Man et al., 2003; Okishio et al., 2005; Won et al., 2002) to avoid overstretching the tissue. With this modification in place, positive concentration-response curves were obtained. Reliable and replicable results were observed during infection and in control mice. This demonstrated the importance of allowing tissue to go back to resting tension before re-stimulating with the next concentration of ACh in order to allow it to re-align its myosin and actin filaments responsible for driving contractions (Ghez, 1991). This showed that overstretched tissue loses tension due to fatigue, in turn yielding attenuated responses to cholinergic stimulation as seen when tissue was stretched to a load of 9.9mN.

Another challenge to overcome in establishing a suitable method to study isometric tension was to understand how tissue area incorporated in force measurements was attained. Zhao et al., 2003 used force per cross-sectional area, where "cross-sectional area (A) = mass (g) × 0.726 / [density (g/ml) × length (cm)], where the mass was corrected for the density of smooth muscle (1.056 g/ml) and the contribution of the fraction of the circular smooth muscle to the total (0.726) muscle mass" as formulated by Zhao et al., 2001. Difficulties in understanding this formula stemmed from the fact that the formula does not results in cm^2 as published (Zhao et al., 2003) but in ml/cm. Another problem posed by this formula is that each tissue is unique and would therefore expect variability in density. This formula appears to ignore that density and circular smooth muscle mass can be altered during infection

due to inflammation—an observation that was made during these experimentations. Therefore these parameters should not be standardized. Due to lack of understanding this formula, it was not applied, and tension results were not presented as mN/cm^2. Further literature search revealed that data might be presented normalized to tissue weight. As the weight of the tissue clearly varied depending on infection and mouse strain used, tension was expressed as mN/mg, as previously shown (Won et al., 2002).

Nippostrongylus brasiliensis infected IL-4Rα-deficient mice tissue contraction

(Part 3)

NB.4.1 Summary

Background

GI transit is initially decreased in *N. brasiliensis* infected small intestine, however it progressively increases during the course of infection (Farmer, 1981). This increase in intestinal transit plays an active role in increased worm expulsion (Collins, 1996; Vallance & Collins, 1998). The stimulated peristaltic activity is characterized by hypercontractile force generated by intestinal longitudinal smooth muscles (Vallance et al., 1997), altered neuronal excitability (Palmer et al., 1998) and neurotransmitter release (Venkova et al., 1999). These responses are modulated by interactions with immune cells including T cells (Collins, 1996; Vallance & Collins, 1998), eosinophils (Vallance et al., 1999a), and mast cells (Stead et al., 1987). In addition to the hypercontractile response, both mucus production and the villi "sweeping" activities aid in worm clearance during *N. brasiliensis* infection. The role of IL-13/IL-4Rα/STAT-6 responsiveness has been shown to play a significant role in helminth infection, with STAT-6-deficient mice demonstrating reduced smooth muscle hypercontractile responses (Akiho et al., 2002). The possible role of IL-4Rα-responsive non-hematopoietic target cells during *N. brasiliensis* infection is less well defined. Therefore, the role of IL-4Rα-responsiveness by smooth muscle cells on intestine jejunum contractility to pharmacological stimulation with ACh during *N. brasiliensis* infection in smooth muscle cell-specific IL-4Rα-deficient mice was investigated in this study.

Methods

IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice were infected with ~750 L3 and analyzed at day 7 or day 10 p.i. compared to non-infected controls. Intestinal worm burden, goblet cell hyperplasia, mucus glycoproteins, histopathology, as well as total antibody production were determined. Concentration-dependent contractile responsiveness to pharmacological stimuli was analyzed from the intestine jejunum.

Results

Infected SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice showed delayed worm clearance and egg expulsion compared to IL-4Rα$^{-/lox}$ mice. Delayed goblet cell hyperplasia was demonstrated in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice compared to IL-4Rα$^{-/lox}$ mice, and

they could not clear the worms completely. As expected, IL-4Rα$^{-/-}$ mice showed impaired goblet cell hyperplasia. Infection resulted in significantly increased ACh-driven intestinal tension in IL-4Rα$^{-/lox}$ mice compared to non-infected IL-4Rα$^{-/lox}$ mice. In contrast, infected IL-4Rα$^{-/-}$ mice jejunum did not demonstrate increased intestinal tension following ACh stimulation. However, infected SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice showed rapid increased but variable and attenuated contractile responses between individual mice and did not reach statistical significance compared to non-infected at day 7 and day 10 p.i.

Conclusion

These results suggest that IL-4Rα-responsiveness by smooth muscle cells is required for optimal cholinergic-induced hypercontractility, and that the absence of IL-4Rα on smooth muscle cells alters the hosts' ability to control infection.

NB.4.2 Results

N. brasiliensis-*infection in IL-4Rα-deficient mice*

IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with ~750 L3. At 7 days and 10 days p.i. parasitological and immunological parameters were determined from the intestine. Qualitative analysis of goblet cell hyperplasia was shown in histology with PAS that stains mucus producing cells red [Figure 3.1]. Goblet cell hyperplasia was quantified by counting the number of enlarged mucus-producing goblet cells. At day 7 p.i. IL-4Rα$^{-/lox}$ mice showed significantly increased numbers of enlarged goblet cells compared to SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice [Figure 3.2A]. However, at day 10 p.i. IL-4Rα$^{-/lox}$ and SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice demonstrated equivalent significant goblet cell hyperplasia compared to IL-4Rα$^{-/-}$ mice, suggesting that SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice have delayed goblet cell hyperplasia. As expected, mucus production (intestinal glycoproteins) measured from tissue homogenates revealed a trend of increased mucus production following infection across all groups [Figure 3.2B]. Worm burden was determined to investigate the ability of mice to clear infection. All mouse strains demonstrated increased worm burdens at day 7 p.i. [Figure 3.2C]. IL-4Rα$^{-/lox}$ mice had cleared worms by day 10 p.i., whereas SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice still harbored worms at that time-point. As expected from previous publication by this laboratory (Horsnell et al., 2007), fecal egg counts were significantly high in global IL-4Rα$^{-/-}$ mice when compared to IL-4Rα$^{-/lox}$ or SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice [Figure 3.2D]. These results suggest that smooth muscle cells IL-4Rα is important for controlled egg transit from the host. Together, these results show successful infection.

Neuronal markers following N. brasiliensis-*infection in IL-4Rα-deficient mice*

The roles of CD4+ T cells and neuropeptide SP have been shown to enhance isolated tissue hypercontractility to ACh stimulation (Vallance et al., 1999b; Zhao et al., 2003). Therefore, the activities of both the enteric immune and the enteric nervous systems may be critical for infection clearance. In order to determine protein expression of immune and neuronal substances in *N. brasiliensis* ACh-induced tension, tissue sections were stained with immunological and neurological markers. Sections were double-stained with anti-CD3 (shown in purple staining) in order to determine proximal relationship of T cells and neurological substances

calbindin and SP (both shown in brown staining) at day 7 p.i. Calbindin identifies calcium-binding proteins in the intestine, and is generally expressed in intrinsic primary afferent neurons (IPANs) (Furness et al., 1990) that are found embedded in the intestine and do not innervate the brain via the vagus nerve (Furness, 2004). These neurons enable the intestine to function as a "mini-brain" following injury or infection.

SP is a neuropeptide that functions as both a neurotransmitter and a neuromodulator. It plays a role in pain perception when released from terminals of sensory nerve fibers of muscles involved in neurogenic inflammation to promote wound healing (Donkin et al., 2007). In the mucosae it may be expressed in epithelial cells and found in neurons of the mucous. SP expression may be elevated following *N. brasiliensis* infection as a result of scaring that occurs due to worms clinging to the intestine and feeding from the host. CD3 positive staining was expressed in all mouse groups in the crypts, villi, and lamina propria, but not in the muscularis [Figure 3.3A-B, Table 3.3]. Calbindin positive staining was found to be expressed in neuronal fibers of all mouse strains [Figure 3.3A], while SP was expressed in neuronal fibers and neuroendocrine cells/enterocytes of all mouse strains [Figure 3.3B]. These qualitative analyses demonstrate the presence of the investigated neurological markers, even in the absence of the IL-4Rα during *N. brasiliensis* infection.

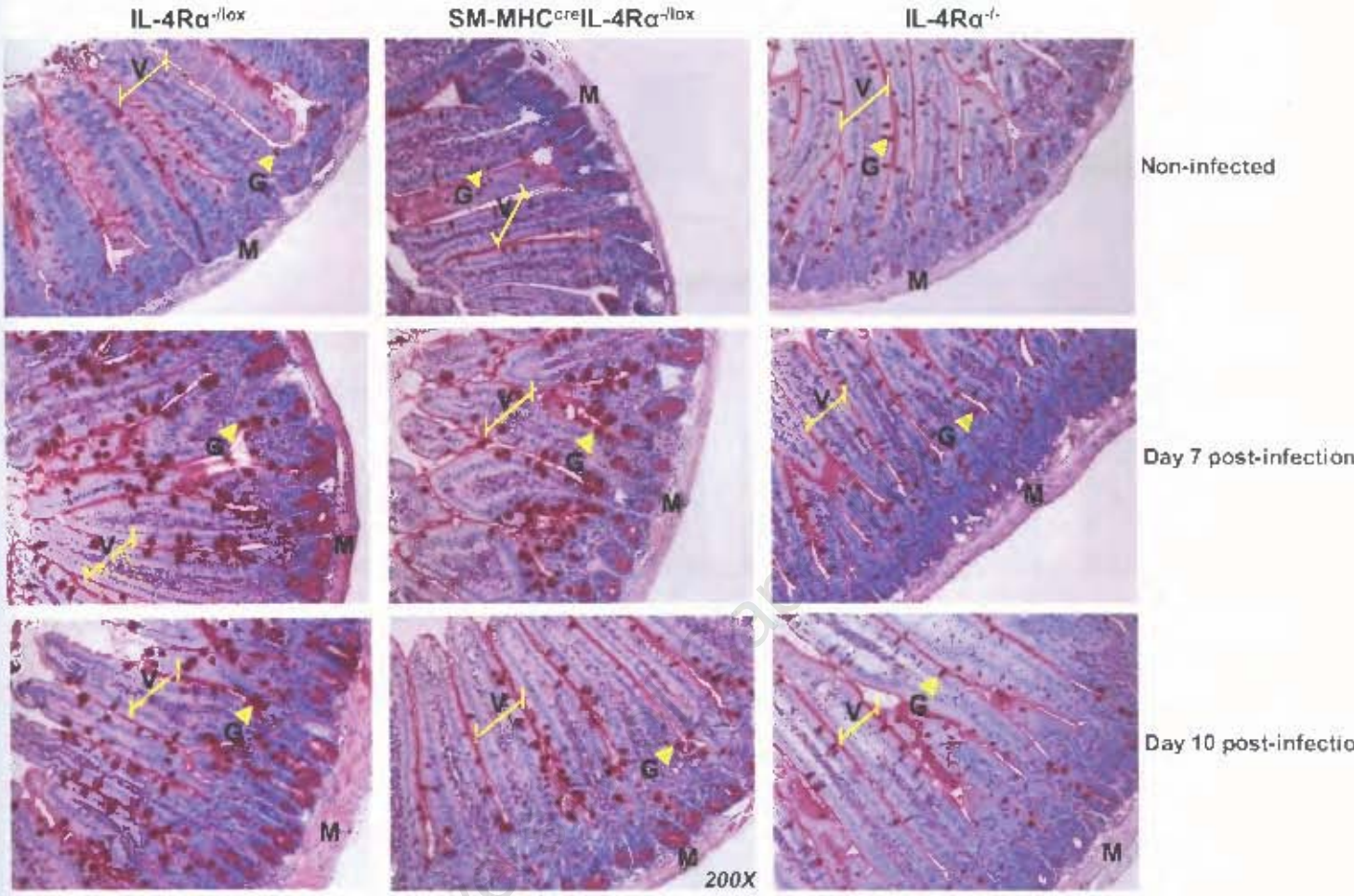


Figure 3.1

The role of IL-4Rα on intestinal histological goblet cell expression following N. brasiliensis *infection*

Mucus-producing goblet cells were visualized using periodic-acid Schiff reagent staining on non-infected and *N. brasiliensis* infected IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice jejunum (n=4) (200X magnification). M: Muscularis, G: Goblet cells [shown by arrow heads], V: Villus [shown by bordered lines]). Figure is a representative of 4 independent experiments with 4 sections per mouse tissue analyzed.

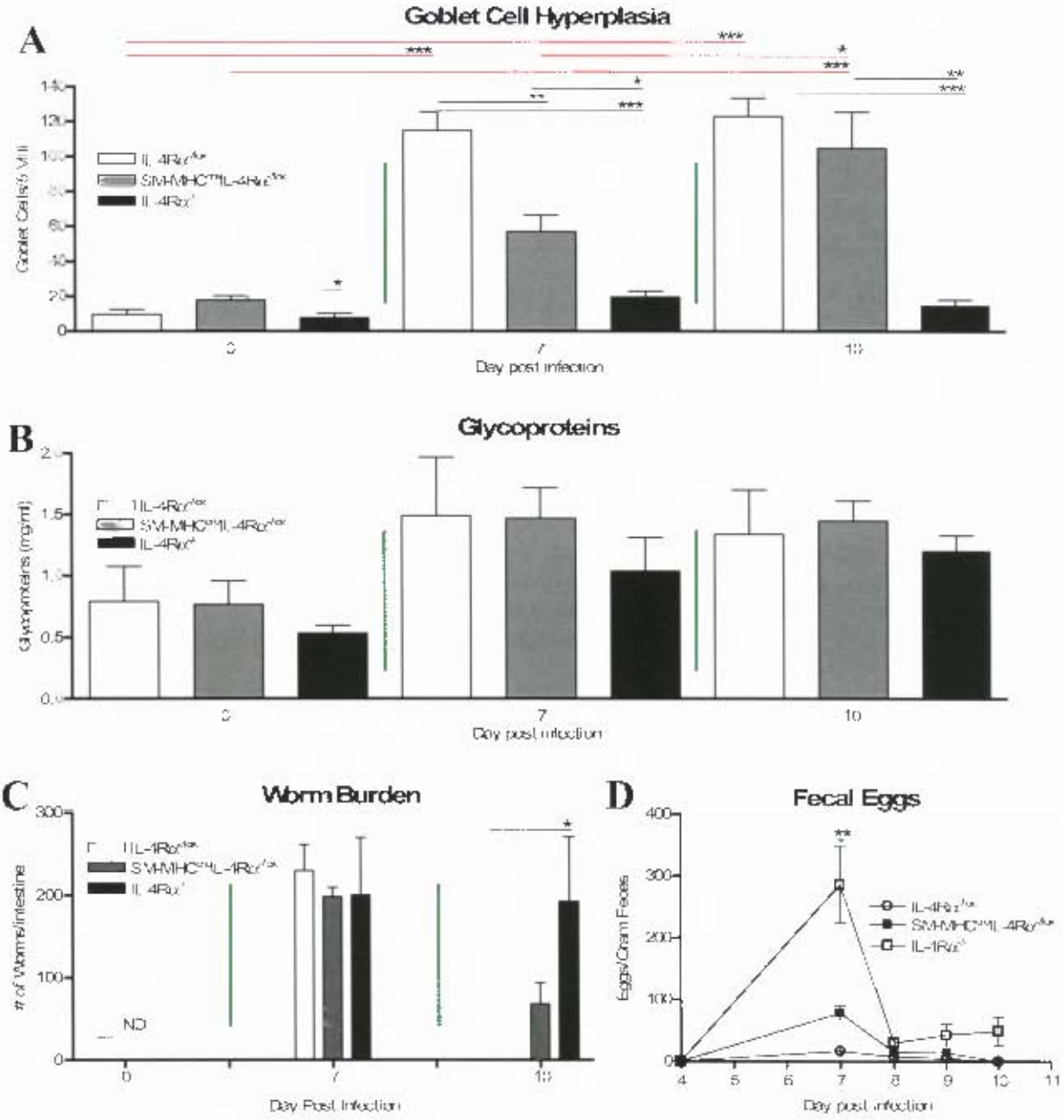


Figure 3.2

Nippostrongylus brasiliensis-*infection in IL-4Rα-deficient mice*

(A) Goblet cell hyperplasia: Mucus-producing goblet cells were visualized using periodic-acid Schiff's reagent staining on the jejunum of non-infected and *N. brasiliensis* infected IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice (n=4). The number of hyperplasic goblet cells per five villi was calculated, counted from 4 tissue sections isolated from 4 individual mice to a total of 20 villi. **(B)** Glycoproteins: Mucus glycoprotein production was determined from individual tissue homogenates by periodic-acid Schiff's assay and expressed in mg/ml. **(C)** Tissue worm burden: Worm burden was established by counting worms in whole intestines removed from infected mice. **(D)** Fecal egg transit: Egg production was assessed in mice fecal pellets following infection daily from day 4 p.i. using the modified McMaster technique. Results were compared between treatment conditions and across strains. Data (mean±SEM) are representative of 2 independent experiments. ANOVA * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

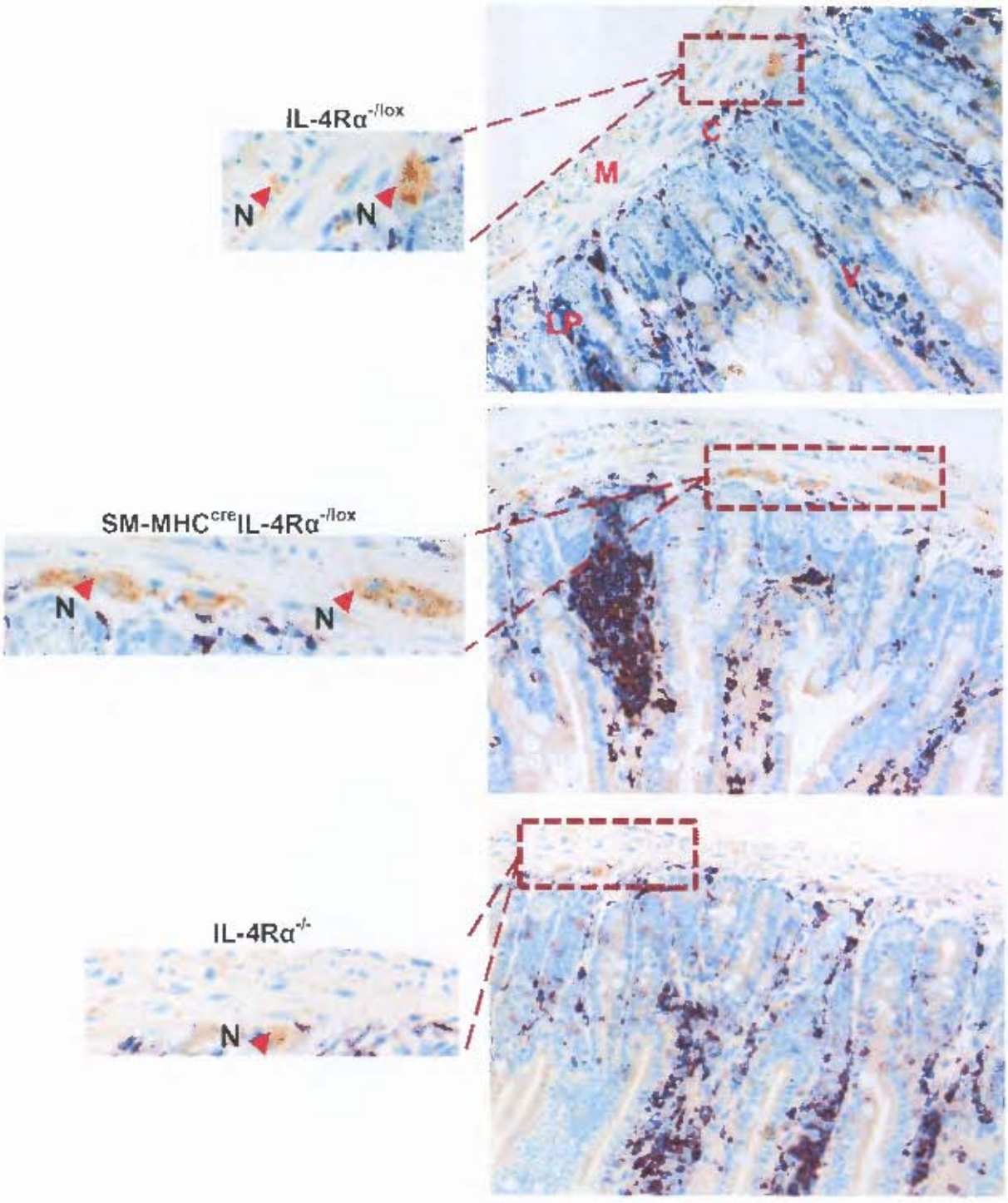


Figure 3.3A

The role of IL-4Rα on T-cells and calbindin expression following N. brasiliensis*-infection*

Histological sections from day 7 p.i. IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice jejunum (n=4) were stained with anti-CD3 (**Purple**) and anti-calbindin (**Brown**). CD3+ staining was expressed in the mucosae, whereas calbindin was expressed in the muscularis. N: neuronal fibers, M: muscularis, C: crypts, V: villi, LP: lamina propria. (200X magnification). Figure is a representation of 4 independent experiments where 4 sections of each isolated mouse jejunum were analyzed.

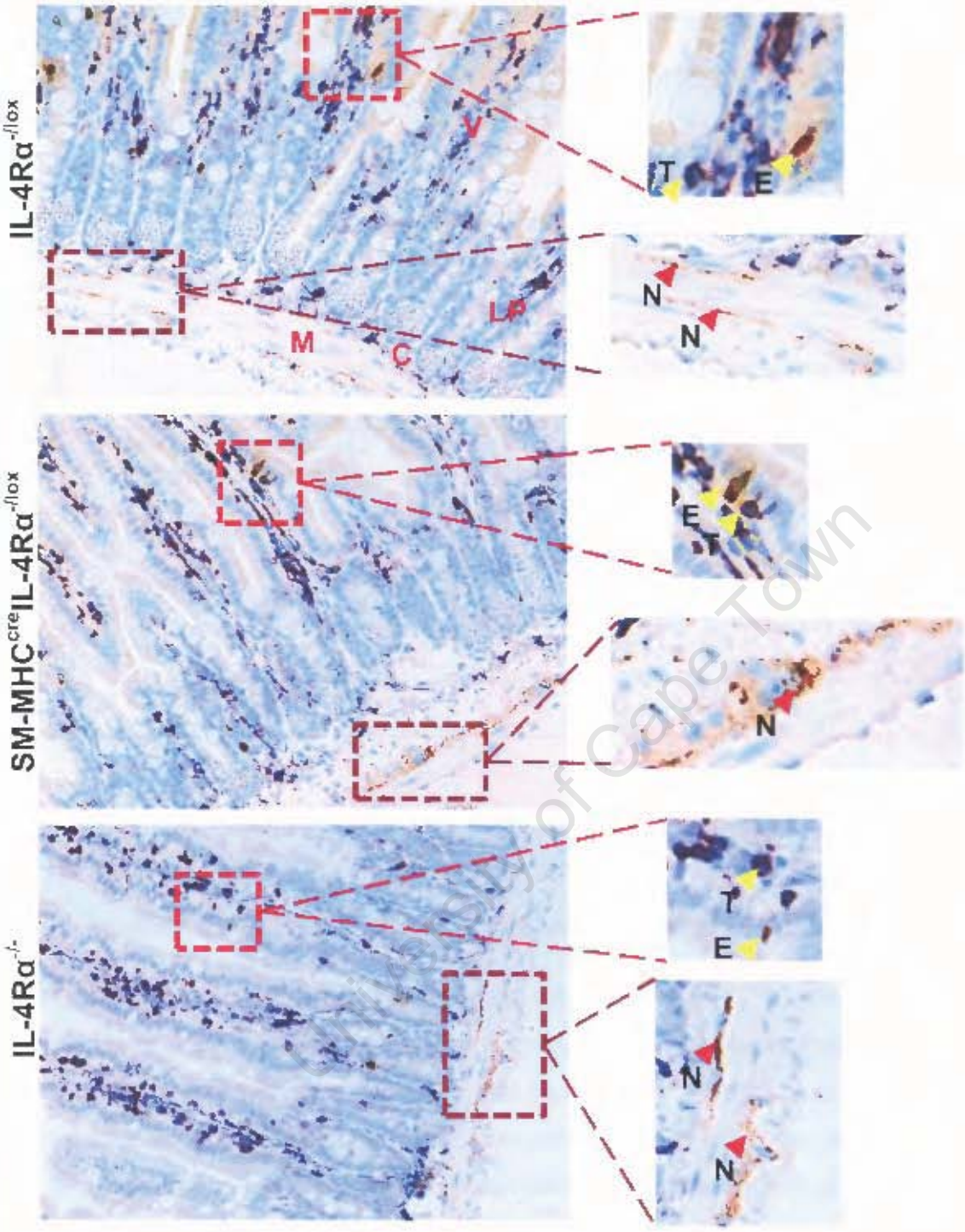


Figure 3.3B

The role of IL-4Rα on T-cells and substance P expression following N. brasiliensis*-infection*

Histological sections from day 7 p.i. IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice jejunum (n=4) were stained with anti-CD3 (**Purple**) and anti-substance P (Brown). CD3+ staining was expressed in the mucosae, whereas substance P was expressed in the muscularis and mucosae. N: neuronal fibers, E: neuroendocrine cells/enterocytes, T: T-cells, M: muscularis, C: crypts, V: villi, LP: lamina propria. (200X magnification). Figure is a representation of 4 independent experiments where 4 sections of each isolated mouse jejunum were analyzed.

		Calbindin	SP	CD3+	Calbindin	SP	CD3+	Calbindin	SP	CD3+
		IL-4Rα$^{-/lox}$			SM-MHC^{cre}IL-4Rα$^{-/lox}$			IL-4Rα$^{-/-}$		
Cell type	T-cells			√			√			√
	Neuronal fibers	√	√		√	√		√	√	
	Neuroendocrine cells/enterocytes		√			√			√	
Intestine area	***Muscularis***	√	√		√	√		√	√	
	Crypts			√			√			√
	Villi		√	√		√	√		√	√
	Lamina propria			√			√			√

Table 3.3

Expression of T-cells, calbindin and substance P following N. brasiliensis*-infection*

Histological sections from day 7 p.i. IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice jejunum (n=4) were stained with anti-CD3, anti-calbindin, and anti-substance P. CD3+ staining was expressed in the mucosae, calbindin was expressed in the muscularis, whereas substance P was expressed in the muscularis and mucosae. SP: substance P. Figure is a representation of 4 independent experiments where 4 sections of each isolated mouse jejunum were analyzed.

Physiological contraction studies in N. brasiliensis*-infected IL-4Rα-deficient mice*

In order to determine if *N. brasiliensis* induced tissue inflammation influenced tissue weight, 1cm long pieces of the jejunum were weighed and results were compared across strains (IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$) and across treatment conditions (non-infected and infected). Infection with *N. brasiliensis* resulted in intestine tissue weight increases in all mouse groups. Statistical significance was reached by IL-4Rα$^{-/-}$ mice at day 7 and day 10 p.i. and by SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice at day 10 p.i. when compared to their non-infected control groups [Figure 3.4A].

Following tissue weight measurements, the jejunum was stimulated with 50mM KCl in order to confirm viability of tissue preparation. Tension increases were observed at day 10 p.i. among IL-4Rα$^{-/lox}$ and SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice compared to their non-infected control groups, while IL-4Rα$^{-/-}$ mice demonstrated no changes in tension following infection [Figure 3.4B]. The same mouse groups (IL-4Rα$^{-/lox}$ and SM-MHC^{cre}IL-4Rα$^{-/lox}$) demonstrated significant tension increases compared to IL-4Rα$^{-/-}$ mice at day 10 p.i.

Isolated tissue that responded with tension increase to KCl stimulation was used to determine the role of IL-4Rα-responsiveness on cholinergic smooth muscle-mediated hypercontractility. As expected, tissue from infected IL-4Rα$^{-/lox}$ mice showed increased tension at day 10 p.i. and not at day 7 p.i. compared to non-infected controls [Figure 3.5]. Tissue from infected SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice demonstrated variable concentration-dependent tension increases in response to ACh stimulation at both day 7 and day 10 p.i. [Figure 3.5]. These responses were attenuated compared to IL-4Rα$^{-/lox}$ mice, and did not reach statistical significance compared to their SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice non-infected controls. Tissue from IL-4Rα$^{-/-}$ mice did not demonstrate increased tension in response to ACh following infection. Taken together, these results demonstrate that smooth muscle cells IL-4Rα is required for enhanced cholinergic tension, thus confirming a direct role of IL-4Rα responsiveness for ACh-induced intestine hypercontractility.

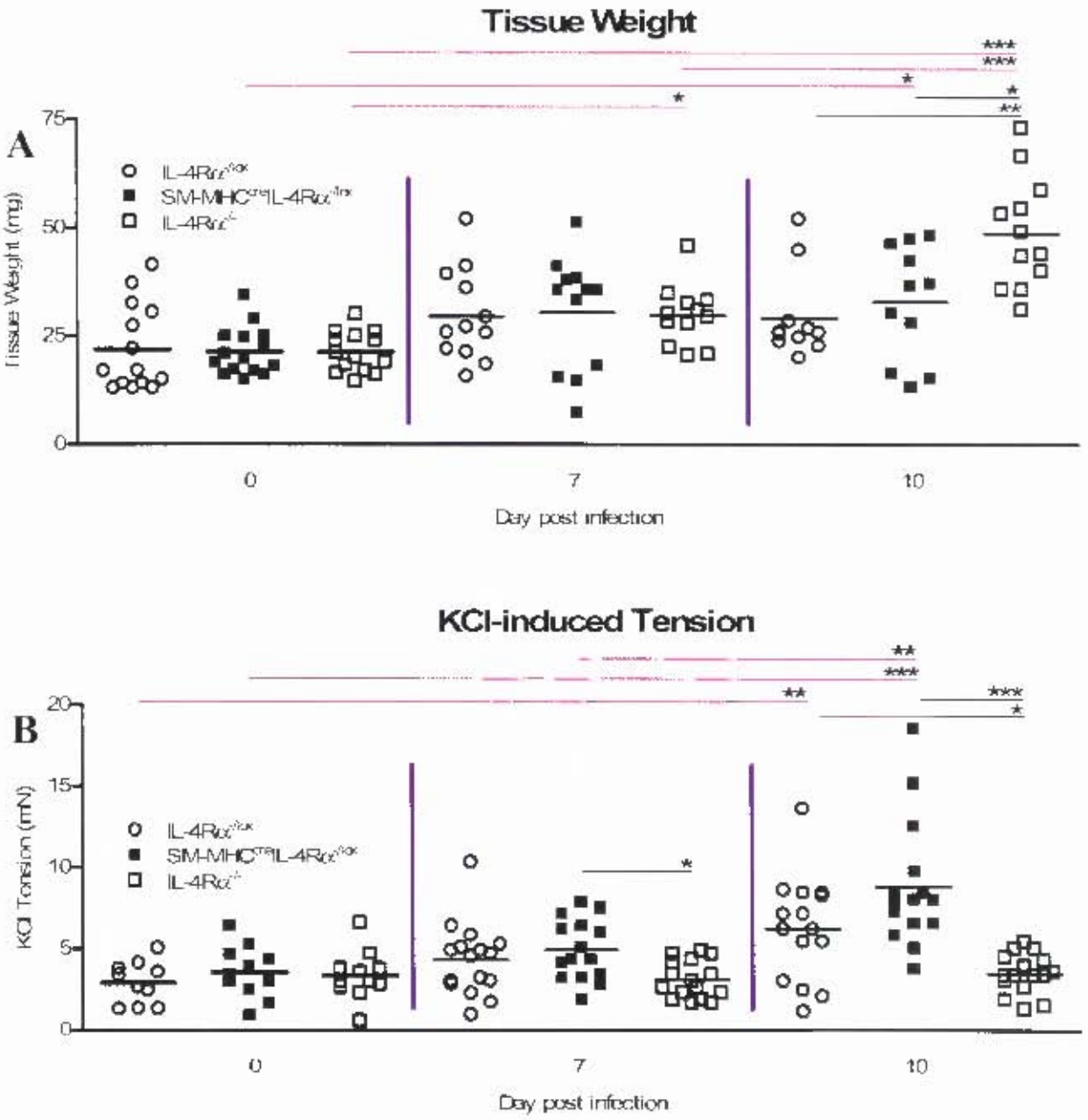


Figure 3.4

The role of IL-4Rα on N. brasiliensis *infected isolated intestine tissue weight and KCl-induced hypercontractility*

(A) Tissue weight: Non-infected and *N. brasiliensis* infected jejunum from IL-4Rα$^{fl/ox}$, SM-MHC^{cre}IL-4Rα$^{fl/ox}$, and IL-4Rα$^{-/-}$ mice (n>10) tested on day 7 and day 10 p.i. was weighed on an analytic scale. **(B)** KCl-induced tension: Tissue was then challenged with 50mM KCl for isometric contractile responses. Data (mean±SEM) are pooled from 3 independent experiments, ANOVA or t-test * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

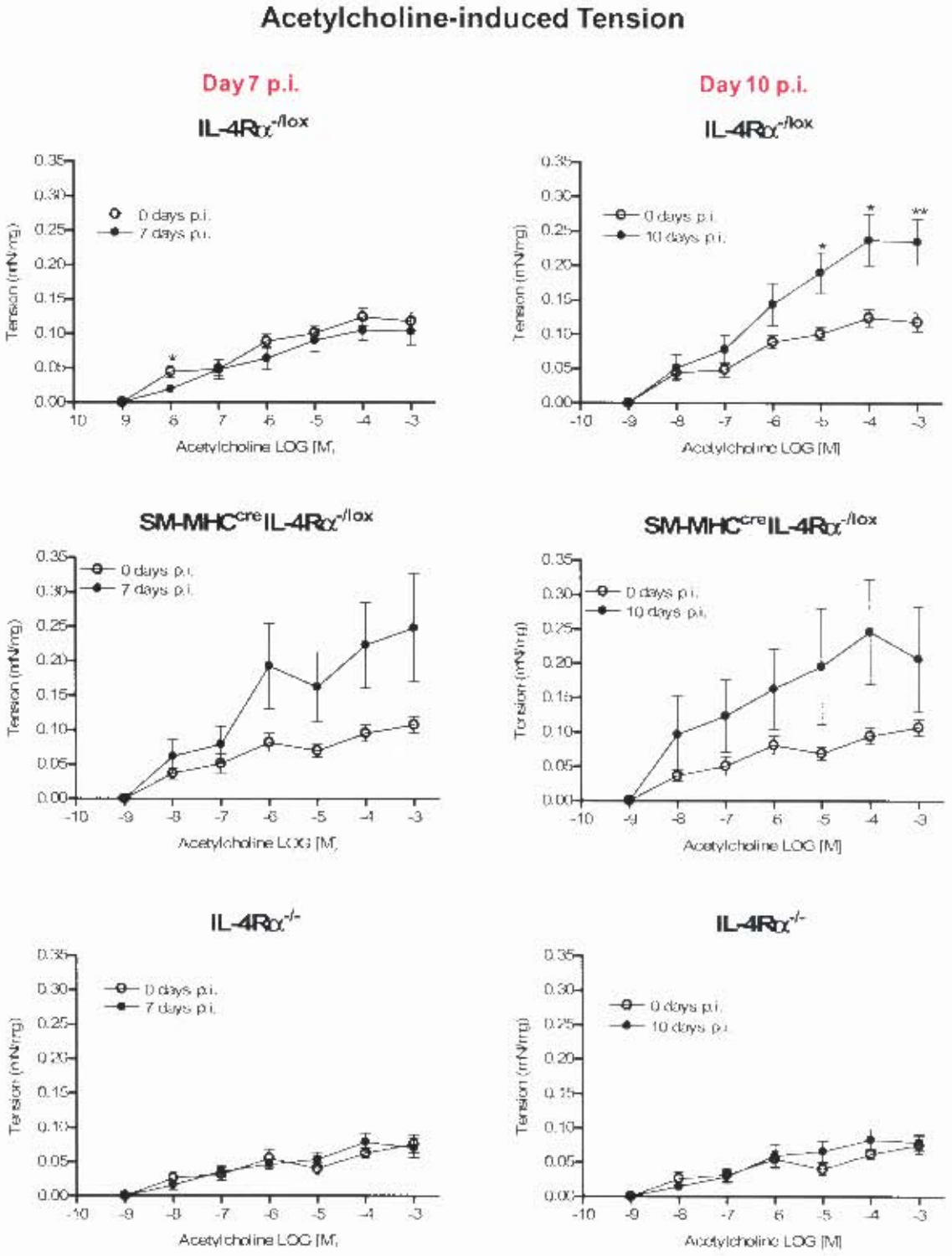


Figure 3.5

The role of IL-4Rα on N. brasiliensis *infected isolated intestine tissue contractility*

Acetylcholine-induced tension: Non-infected and *N. brasiliensis* infected jejunum from IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice (n>10) tested on day 7 and day 10 p.i. was weighed on an analytic scale, challenged with 50mM KCl for isometric contractile responses followed by varying concentrations of ACh. Data (mean±SEM) are pooled from 3 independent experiments, ANOVA or t-test * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Schistosoma mansoni-induced IL-4Rα-dependent smooth muscle cells contraction

(Part 4)

SM.4.1 Summary

Background

S. mansoni is a blood fluke that has adult worms mate in the portal circulation of the liver within small vessels. Worms eventually migrate against blood flow to the tributaries of the inferior mesenteric vein where females deposit eggs. The eggs penetrate the vascular walls in order to reach the intestine wall. Eggs pierce the mucosal lining of the intestine to reach the intestine lumen. Inflammatory responses to eggs antigen results in granuloma formation around the egg (Herbert, 2004). *S. mansoni* infection dependent intestinal pathology is largely a result of parasitic eggs traversing the intestine. Egg transit leads to tissue damage associated with increased intestine tissue contractility possibly caused by trophic and functional changes at smooth muscle cell level (Moreels et al., 2001a). Intestinal hypercontractile responses to helminth infections have been widely demonstrated to be dependent on host IL-13/IL-4Rα/STAT-6 responsiveness (Akiho et al., 2002). The role of IL-13/IL-4Rα/STAT-6 expression in *S. mansoni* infection-induced intestinal hypercontractility has not been previously demonstrated. In the study presented here, we investigated how both global deletion of IL-4Rα and smooth muscle cell specific disruption of IL-4Rα expression affects intestinal cholinergic hypercontractility in *S. mansoni* infected mice. The role of IL-4Rα-responsiveness by smooth muscle cells on intestine tissue contractility to cholinergic stimuli during comparative infection studies in smooth muscle cell-specific IL-4Rα-deficient mice and their respective controls is investigated.

Methods

IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice were infected with 80 cercariae and analyzed at 8 weeks p.i. for the disease phenotype by measuring egg burden, histopathology, as well as total and SEA-specific antibody production. Concentration-dependent contractile responsiveness to cholinergic stimuli was analyzed from intestine ileum.

Results

As expected from previously published results, control IL-4Rα$^{-/lox}$ mice responded with a predominant type 2 antibody response, whereas IL-4Rα$^{-/-}$ mice showed a shift to a type 1 response with increased levels of SEA-specific IgG2a and impaired IgE production. Infected SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice showed a similar type 2 antibody response as control IL-4Rα$^{-/lox}$ mice. SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice showed egg retention and intestine pathology. Reduced egg expulsion was accompanied by impaired IL-4/IL-13-mediated hypercontractile intestine responses following *ex vivo* cholinergic stimulation, which were attenuated in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice and abrogated in global IL-4Rα$^{-/-}$ mice.

Conclusion

Together, it is concluded that IL-4Rα-responsiveness by smooth muscle cells and subsequent IL-4 and IL-13-mediated hypercontractility via the IL-4Rα-chain is required for host protection during acute schistosomiasis to efficiently expel *S. mansoni* eggs, and to prevent premature mortality.

SM.4.2 Results

S. mansoni-infection in IL-4Rα-deficient mice

IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice were infected with 80 cercariae. At 8 weeks p.i. liver parasitological and immunological parameters were determined. SEA-specific IgG2a characteristic for type 1 immune responses and total IgE characteristic for type 2 immune responses were determined from the blood plasma of infected mice. Figure 4.1A shows a predominant type 2 antibody response in control IL-4Rα$^{-/lox}$ and a predominant type 1 antibody response in global IL-4Rα$^{-/-}$ mice. SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice showed a similar predominant type 2 antibody response compared to control IL-4Rα$^{-/lox}$ mice during *S. mansoni* infection. Eggs produced by the worm and released into the blood stream can get trapped in the organs, particularly the liver and the intestine, where they try to reach the lumen to be expelled. This lead to granuloma formation by the host around the eggs, as shown for the ileum [Figure 4.1B], causing chronic organ pathology including fibrosis. Following infection, all mouse strains developed muscularis mucosae hyperplasia associated with egg deposits [Figure 4.1C]. Egg counts were conducted on both liver and intestine tissues to investigate tissue eggs retention, revealing significantly low egg numbers in both IL-4Rα$^{-/lox}$ and SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice compared to IL-4Rα$^{-/-}$ mice [Figure 4.1D]. Fecal eggs were quantified to determine if tissue egg retention influenced egg transit, showing significantly low egg burdens among both SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice compared to control IL-4Rα$^{-/lox}$ mice [Figure 4.1E]. Taken together, these results demonstrate that SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice have unaltered egg retention, but impaired egg expulsion, suggesting that IL-4Rα responsiveness in smooth muscle cells is important for optimal egg expulsion.

Intestine tissue weight in S. mansoni-infected IL-4Rα deficient mice

Intestine ileum tissue weight has been used to normalize stimulus induced isometric tension contractile responses (Moreels et al., 2001b) as *S. mansoni* egg-induced intestine inflammation may influence tissue contractility. Tissue weights across strains (IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$) and across treatment conditions (non-infected, infected) were investigated at 8 weeks p.i. where efficient egg production is present. 1cm long pieces of the ileum were collected from mice and weighed on an analytic scale. Tissue weight of non-infected control IL-4Rα$^{-/lox}$

mice was significantly higher compared to non-infected SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice [Figure 4.2D], when pooling the two independent experiments. Importantly, infection tended to increase tissue weight in both experiments with some variability, and statistical significance found in the pooled experiments among SM-MHC^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice compared to their non-infected controls [Figure 4.2A-C]. However, the weight at 8 weeks p.i. between the three infected mouse strains was similar [Figure 4.2E]. These observed weight increases are in line with the intestinal inflammation caused by the *S. mansoni* eggs during traversing to the intestine lumen.

KCl contraction studies in S. mansoni-infected IL-4Rα deficient mice

KCl has been used to test viability of tissue preparations (Stengel et al., 2002) in experiments before challenges with a stimulus of interest. Therefore we used this method to test the ability of intestine tissue (ileum) to contract to 50mM KCl, to determine tissue viability. Tension increases in response to KCl stimulation was found in all three mouse strains [Figure 4.3A-C] with some variability but clear statistical significance, particularly in the pooled data representation. This was most prominent in infected control IL-4Rα$^{-/lox}$ and SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice, with the least increase in infected IL-4Rα$^{-/-}$ mice [Figure 4.3E]. As expected, non-infected strains showed low contractile responses to KCl [Figure 4.3D]. Together, these results demonstrated that the tissues were viable with increased non-cholinergic contraction during infection with *S. mansoni*. The similar tension increase from tissue, isolated from infected control IL-4Rα$^{-/lox}$ mice and SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice but reduced tension in global IL-4Rα$^{-/-}$ mice may further suggest that absence of IL-4Rα on smooth muscle cell does not influence KCl-induced tension, whereas global IL-4Rα deficiency may have an attenuating effect to KCl-induced tension.

Acetylcholine contraction studies in S. mansoni-infected IL-4Rα deficient mice

The involvement of IL-4Rα responsiveness on cholinergic smooth muscle mediated contractions using *sv*.non-cumulative method was investigated on isolated ileum following tissue weights measurement and KCl stimulation. Data were analyzed as absolute force (mN) [Figure 4.4] and as force normalized by tissue weight (mN/mg) to correct for the observed weight differences [Figure 4.5]. As expected, tissues from non-infected mice of all groups responded with a similar ACh concentration-

dependent tension increase [Figure 4.4D]. This suggests that IL-4Rα responsiveness has no influence on ACh responses from non-infected tissue. Tissue from infected mice showed strikingly increased ACh concentration-dependent tension compared to non-infected controls. This hypercontractility was highest in control IL-4Rα$^{-/lox}$ mice, attenuated in SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice and strikingly reduced in global IL-4Rα$^{-/-}$ mice [Figure 4.4A-C]. When tissue weights were incorporated (mN/mg), a similar response was observed between the different mouse strains [Figure 4.5A-C] due to the fact that the tissue weights between the infected strains were similar [Figure 4.2]. Together, these results demonstrate that *S. mansoni*-induced contractility *in vivo* is IL-4Rα-dependent and leads to muscarinic receptor-mediated hypercontractility. The results further suggested that IL-4Rα signaling in smooth muscle cells directly induces this hypercontractility associated with maximal *Schistosoma* egg expulsion.

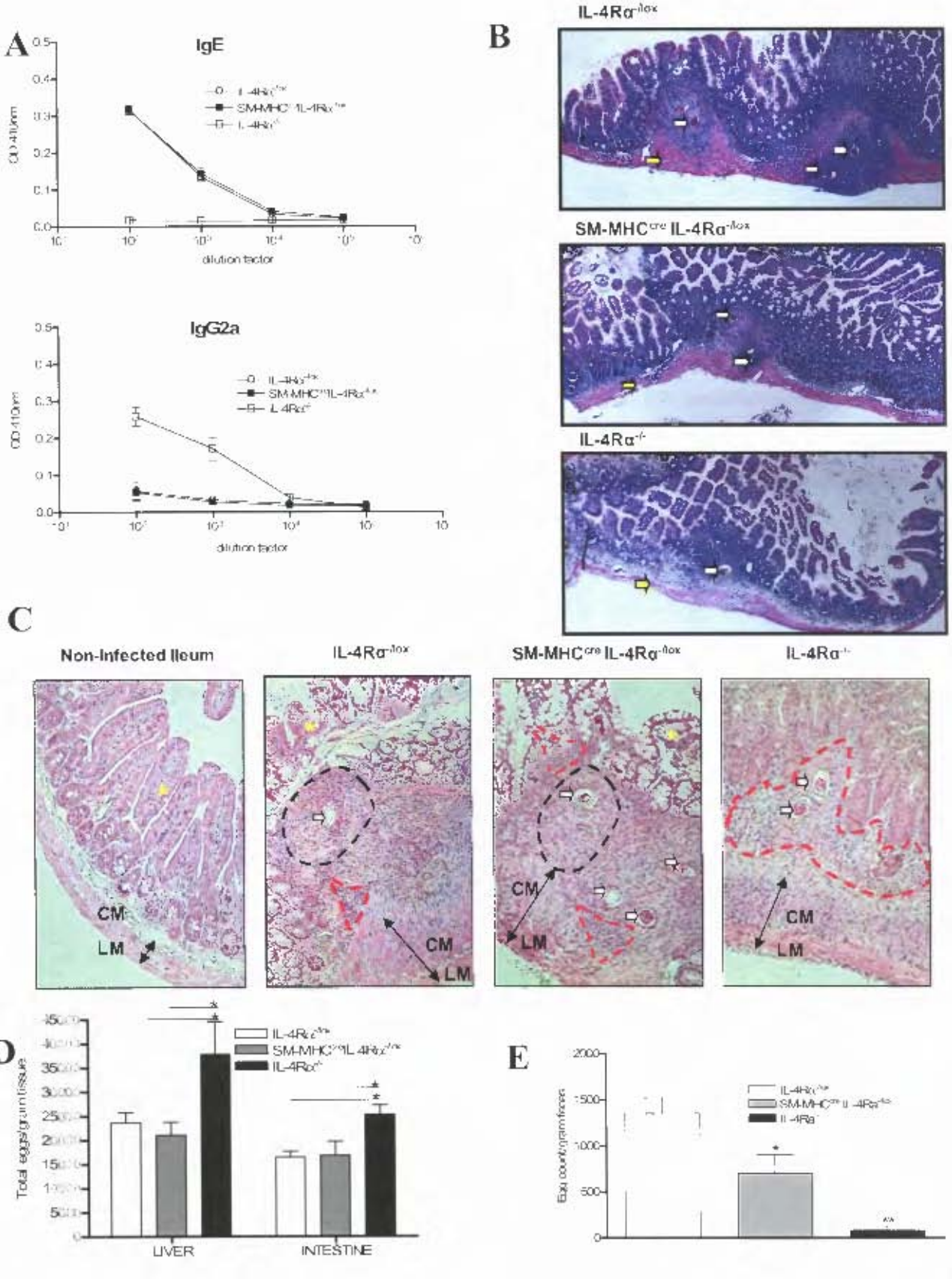

A
IgE
IgG2a
dilution factor
B
IL-4Rα-/lox
SM-MHCcre IL-4Rα-/lox
IL-4Rα-/-
C
Non-Infected Ileum
IL-4Rα-/lox
SM-MHCcre IL-4Rα-/lox
IL-4Rα-/-
CM
LM
D
Total eggs/gram tissue
LIVER
INTESTINE
E
Egg count/gram feces

Figure 4.1

Figure 4.1

Schistosoma mansoni-*infection in IL-4Rα-deficient mice*

(A) Antibody responses: total IgE antibody production and SEA-specific IgG2a antibody production were determined from sera of 8 week-infected IL-4Rα$^{-/lox}$ (white), SM-MHC^{cre}IL-4Rα$^{-/lox}$ (grey) and IL-4Rα$^{-/-}$ (black). N=4 mice per group. Data (mean±SEM) are representative of 3 independent experiments. *P<0.05; ** *P*<0.001 compared to IL-4Rα$^{-/lox}$ control mice). **(B)** Diffuse inflammatory granulomata: Small intestine sections (H&E, original magnification 40X) with thickening of the muscularis propria (yellow arrow) in the area closely surrounding by eggs (white arrow). **(C)** Intestine granuloma formation: Muscularis propria from the small intestine with enteritis and hyperplasia (H&E, original magnification 100x) indicated with black arrow; circular (CM) and longitudinal (LM) muscle layer; yellow * indicate villi; white arrow indicate egg, lines demarcate examples of granuloma (black) or enteritis (red). **(D)** Tissue egg counts and **(E)** fecal egg output: Eggs from individual mice were counted. Data (mean±SEM) are representative of 2 individual experiments (n=4 mice per group). *P<0.05; ** p<0.01 compared to IL-4Rα$^{-/lox}$ control mice. (Data taken from Marillier et al., 2010).

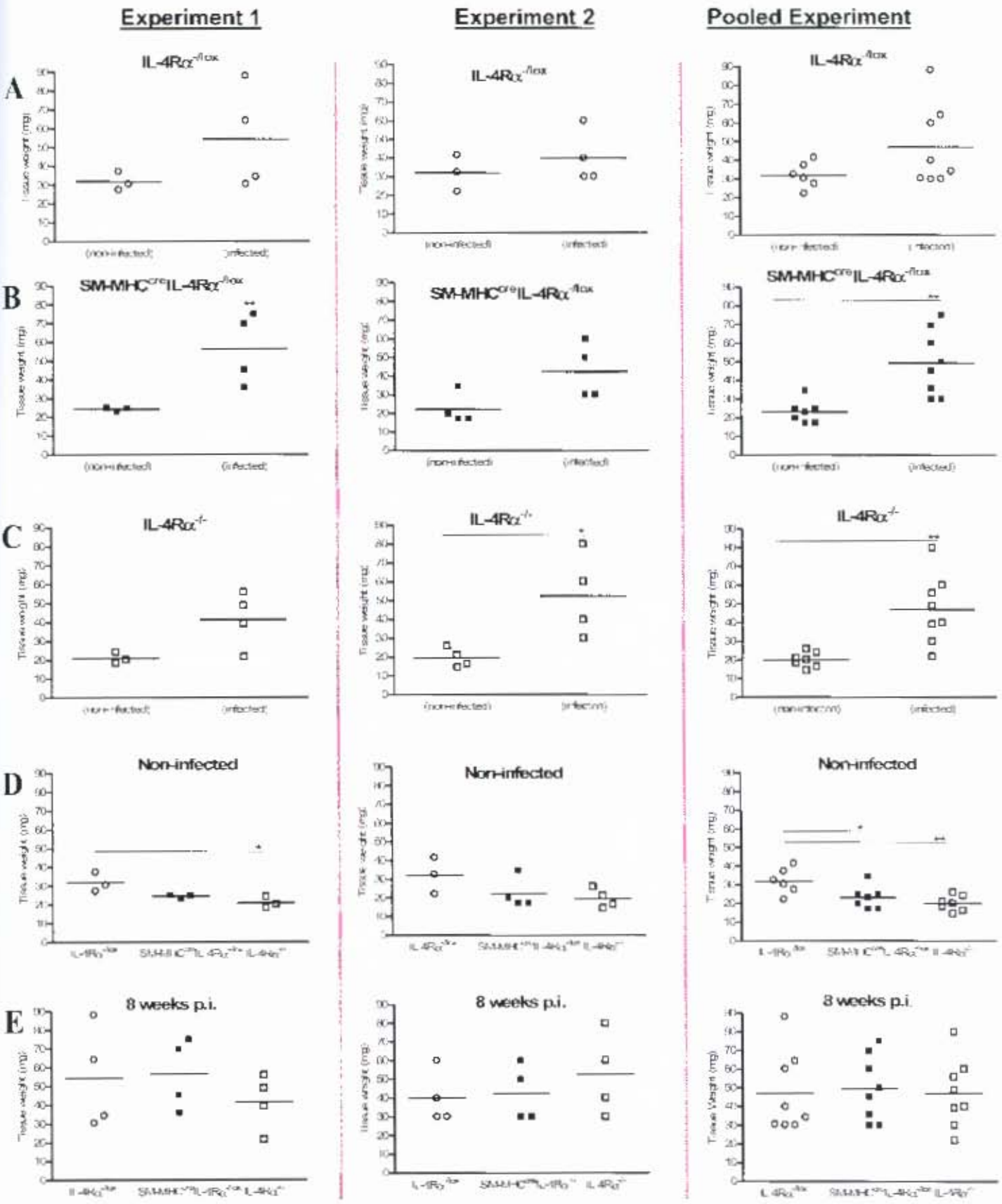

Experiment 1
Experiment 2
Pooled Experiment
A
B
C
D
E
IL-4Rα-/flox
SM-MHCcre IL-4Rα-/flox
IL-4Rα-/-
Non-infected
8 weeks p.i.
Tissue weight (mg)
(non-infected)
(infected)

Figure 4.2

Figure 4.2

The role of IL-4Rα on S. mansoni*-infected isolated intestine tissue weight*

Non-infected (n=3-4) and infected (n=4) IL-4Rα$^{-/lox}$, SM-MHC^{cre}IL-4Rα$^{-/lox}$, and IL-4Rα$^{-/-}$ mice ileum infected with *S. mansoni* were weighed on an analytic scale and results were compared between treatment conditions **(A, B, C)** and across strains **(D, E)**. Tissue weight results are shown as mean±SEM, t-test or ANOVA * $p<0.05$; ** $p<0.01$.

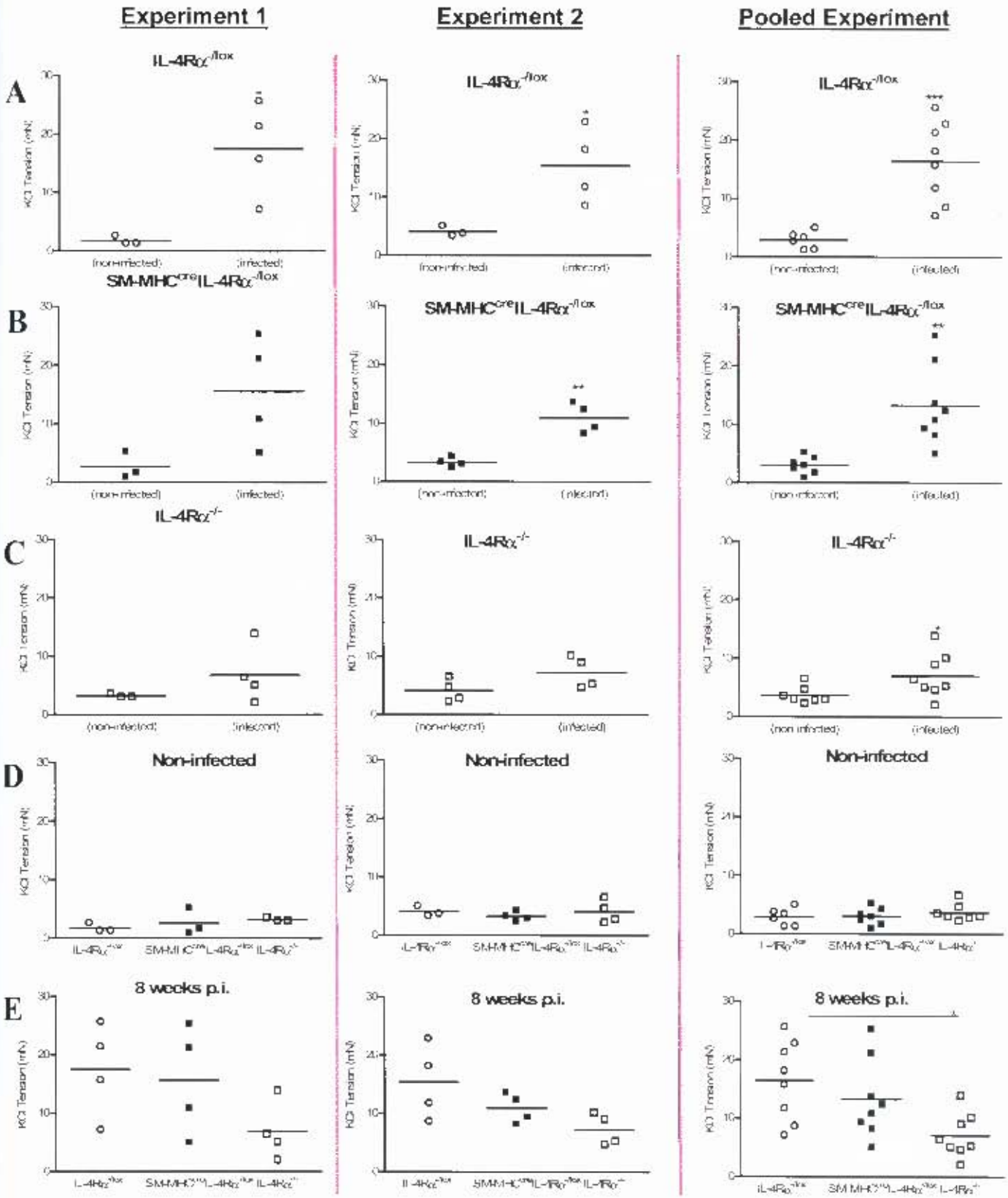

Experiment 1
Experiment 2
Pooled Experiment
A
B
C
D
E
IL-4Rα-/flox
SM-MHCcreIL-4Rα-/flox
IL-4Rα-/-
Non-infected
8 weeks p.i.
KCl Tension (mN)
(non-infected)
(infected)

Figure 4.3

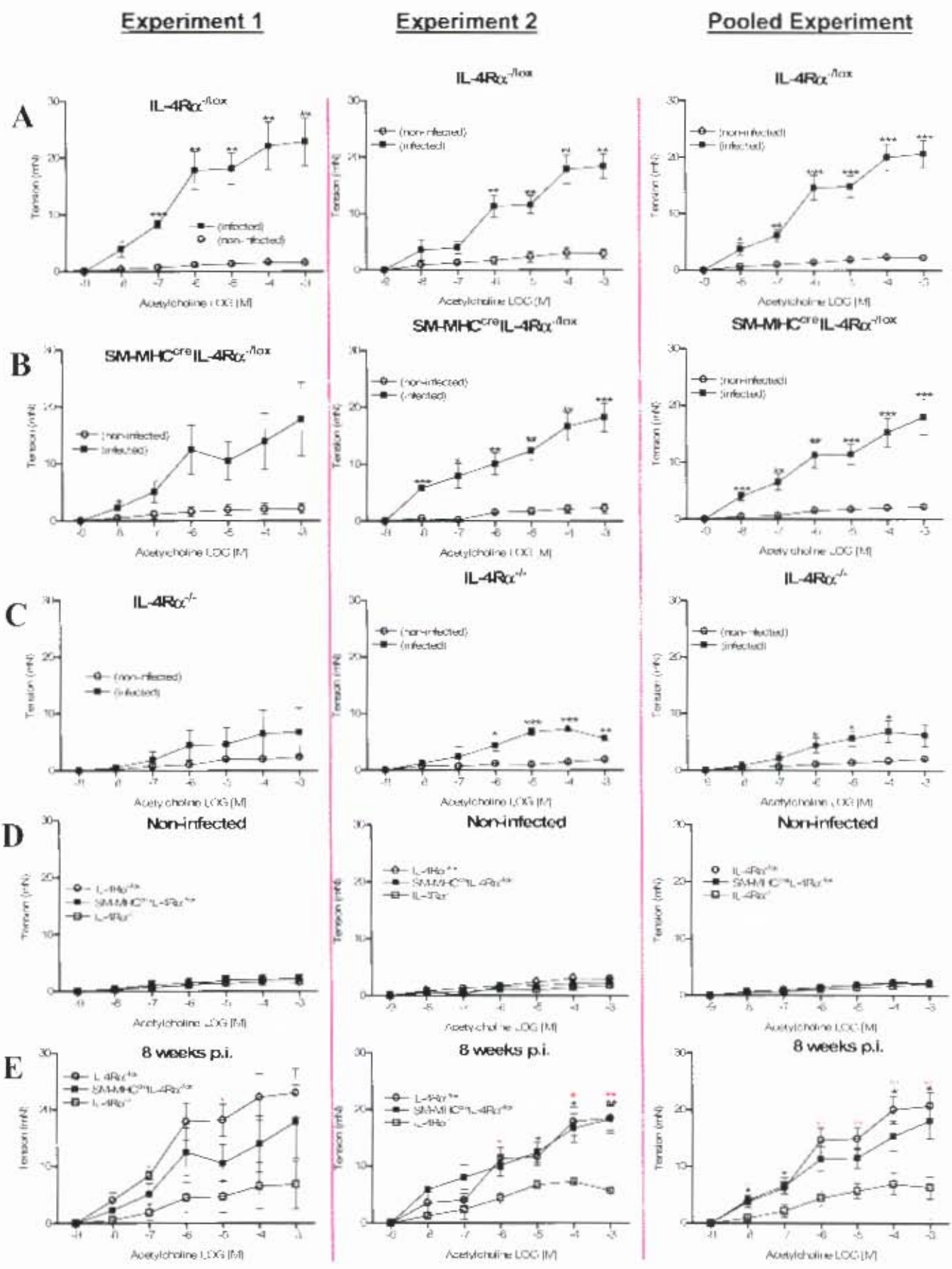

Experiment 1
Experiment 2
Pooled Experiment
A
B
C
D
E
IL-4Rα-/flox
SM-MHCcreIL-4Rα-/flox
IL-4Rα-/-
Non-infected
8 weeks p.i.
(non-infected)
(infected)
Tension (mN)
Acetylcholine LOG [M]

Figure 4.4

Figure 4.4

The role of IL-4Rα on ACh stimulated S. mansoni*-infected isolated intestine tissue contraction expressed as force*

Non-infected (n=3-4) and infected (n=4) IL-4Rα^{-flox}, SM-MHC^{cre}IL-4Rα$^{-/flox}$, and IL-4Rα$^{-/-}$ mice ileum infected with *S. mansoni* were challenged with ACh for isometric contractile responses. Results were compared between treatment conditions **(A, B, C)** and across strains **(D, E)**. Maximum contractile responses evoked by challenges are shown as mean±SEM, t-test or ANOVA * $p<0.05$; ** $p<0.01$; *** $p<0.001$. [IL-4Rα^{-flox} vs. IL-4Rα$^{-/-}$; SM-MHC^{cre}IL-4Rα$^{-/flox}$ vs. IL-4Rα$^{-/-}$].

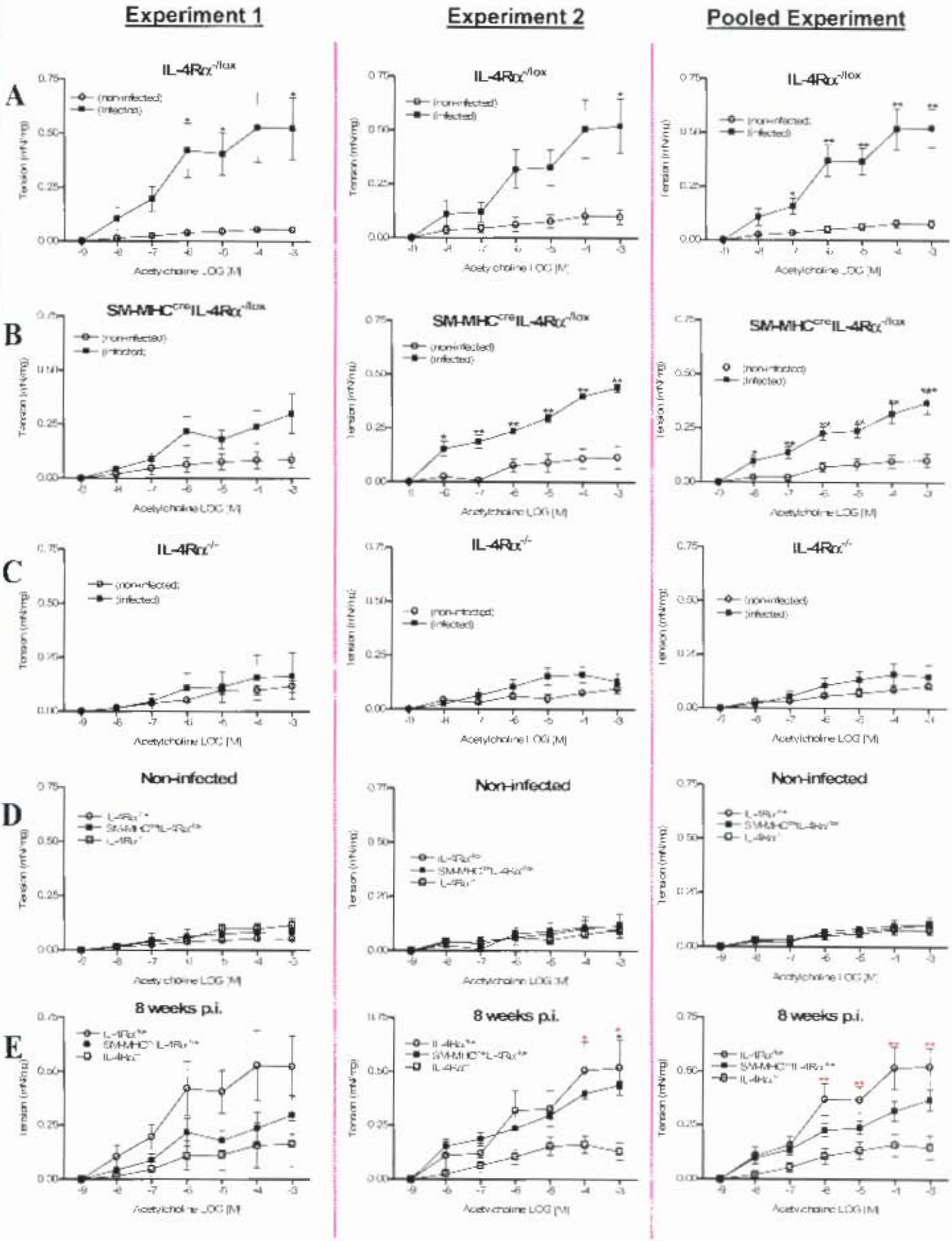

Experiment 1
Experiment 2
Pooled Experiment
A
B
C
D
E
IL-4Rα-/flox
SM-MHCcreIL-4Rα-/flox
IL-4Rα-/-
Non-infected
8 weeks p.i.
(non-infected)
(infected)
Tension (mN/mg)
Acetylcholine LOG [M]

Figure 4.5

Figure 4.5

The role of IL-4Rα on ACh stimulated S. mansoni*-infected isolated intestine tissue contraction expressed as force/tissue weight*

Non-infected (n=3-4) and infected (n=4) IL-4Rα^{flox}, SM-MHC^{cre}IL-4Rα^{flox}, and IL-4Rα$^{-/-}$ mice ileum infected with *S. mansoni* were challenged with ACh for isometric contractile responses. Results were compared between treatment conditions **(A, B, C)** and across strains **(D, E)**. Maximum contractile responses evoked by challenges are shown as mean±SEM, t-test or ANOVA * $p<0.05$; ** $p<0.01$; *** $p<0.001$. [IL-4Rα^{flox} vs. IL-4Rα$^{-/-}$; SM-MHC^{cre}IL-4Rα^{flox} vs. IL-4Rα$^{-/-}$].

Discussion

A bidirectional communication network between the nervous and immune system has been shown with immune products signaling the brain, and the brain in turn responding by modulating immune function and coordinating host defense. Cytokines (e.g. IL-4 and IL-13) participate in this communication between the immune system and central nervous system following infection or injury. Other participants include neuropeptides (e.g. substance P) and neurotransmitters (e.g. ACh) (see picture A).

The role of IL-4Rα-dependent intestinal hypercontractility to cholinergic stimulation in response to *N. brasiliensis* and *S. mansoni*-acute infection was investigated *ex vivo* in comparative studies with global and smooth muscle cell-specific IL-4Rα-deficient mice and their littermate controls. Smooth muscle cell-specific IL-4Rα-deficient mice were investigated, demonstrating the importance of IL-4Rα responsiveness by smooth muscle cells for *N. brasiliensis* and *S. mansoni*-induced intestinal hypercontractility. Intestine tissue from infected SM-MHC^{cre}IL-4Rα$^{-/lox}$ mice demonstrated attenuated cholinergic-mediated hypercontractile responses that aid in egg expulsion to prevent premature mortality during acute schistosomiasis and efficient worm expulsion during *N. brasiliensis* infection.

The ability of both the immune and nervous systems to control smooth muscle hypercontractility has been reported (Matsui et al., 2002; Zhao et al., 2003). Increased Tension increases to ACh stimulation were reported in WT mice infected with *N. brasiliensis.* These responses were attenuated but not abrogated in STAT-6$^{-/-}$ mice, suggesting partial dependence of smooth muscle cells hypercontractility on the IL-4/IL-13/STAT-6 pathway *in vivo* (Zhao et al., 2003). In the absence of M_2 and M_3 receptors in the intestine, longitudinal smooth muscle tissue failed to respond to the ligand carbachol, even at concentrations that evoked maximal contractions in WT mouse tissues, which demonstrated the direct effects of ACh on smooth muscle hypercontractility (Matsui et al., 2002). Moreover, it has been shown by *in vitro* tissue contraction studies that IL-4 and IL-13 promote hypercontractility of ACh-stimulated tissue, and that IL-4 can directly enhance smooth muscle contractility without influence of the enteric nervous system (Zhao et al., 2003). More recently, Horsnell et al., 2007 demonstrated a positive correlation between IL-4Rα mRNA

expression and M_3 receptor mRNA expression as global and smooth muscle cell-specific IL-4Rα-deficient mice showed significantly lower and delayed levels of M_3 receptor mRNA expression compared to control IL-4Rα$^{-/lox}$ mice during *N. brasiliensis* infection (see picture B). This was the first report of IL-4Rα expression having an effect on mAChR expression *in vivo*. As both mutant mouse strains were more susceptible to infection, with delayed worm expulsion and reduced T_H2 cytokine responses, it was hypothesized that IL-4Rα-mediated smooth muscle cell contractility was affected, which might have caused delayed expulsion. What is yet to be explored is if T_H2 cytokines (IL-4 and IL-13) influence neuronal activity, leading to contractility and the mechanisms of action (see picture C).

Significant jejunum tissue weight differences were also observed during *N. brasiliensis* infection. Infected smooth muscle cell-specific IL-4Rα-deficient mice tissue showed increased tissue weight at day 10 p.i. correlating to KCl-induced tension. This result showed that *N. brasiliensis* infection does not only lead to tissue inflammation that enhances weight, but also suggests that inflammation may cause tissue structural alterations that induces non-ligand specific hypercontractility as previously reported during *S. mansoni* infection (Moreels et al., 2001a). A negative correlation was shown between infected IL-4Rα$^{-/-}$ mice tissue weight and KCl-induced tension. This may suggest that worms residing in the intestine lumen may not cause dramatic alterations to the muscle layer of IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice. Any possible tissue structural alterations may be due to IL-4Rα deletion in smooth muscle cell-specific IL-4Rα-deficient mice, and enhanced by *N. brasiliensis* infection. Significant ACh-induced tension increases present at day 7 p.i. in smooth muscle cell-specific IL-4Rα-deficient mice compared to IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice showed that IL-4Rα responsiveness in smooth muscle cells is required for cholinergic-induced hypercontractility.

In this study protein expression of CD3+ T cells in close proximity to calbindin and SP expressing cells of the intestine during infection with *N. brasiliensis* is shown. This result confirmed co-localization of neuro-immunological substances following *N. brasiliensis* infection as contractile responses to SP following *S. mansoni* and *N. brasiliensis* infection have been shown previously (Moreels et al., 2001a; Zhao et al., 2003).

Work from the host laboratory previously demonstrated that smooth muscle cell-specific IL-4Rα-deficient mice have delayed worm expulsion and egg transit associated with a reduction in the ability of the host to trigger type 2 immune responses (Horsnell et al., 2007). This observation was confirmed in this study and expanded by demonstrating that cholinergic hypercontractility is partially dependent on smooth muscle cell-IL-4Rα responsiveness. Although a trend for smooth muscle cell-IL-4Rα dependent intestine hypercontractility was observed following *N. brasiliensis* infection in mice, it was not statistically significant. Indeed, *N. brasiliensis* infection in mice lead to a robust immune response and clearing of the invader within 10 days, which as expected. This was accompanied by a predominant T_H2 response, which was impaired in IL-4Rα$^{-/-}$ mice, as previously shown (Barner et al., 1998). Subsequently, produced IL-4 resulted in a B cell antibody isotype switch to type 2 antibodies, demonstrated by predominant antigen-specific IgG1 and IgE production. IL-4 and IL-13-dependent goblet cell hyperplasia resulted in increased mucus production in the intestine of control mice, which was delayed in smooth muscle cell-specific IL-4Rα-deficient mice and impaired in global IL-4Rα$^{-/-}$ mice. Moreover, at day 7 p.i. smooth muscle cell-specific IL-4Rα-deficient mice demonstrated comparable worm burdens and egg production as IL-4Rα$^{-/lox}$ mice. As previously shown by Horsnell et al., 2007, worm expulsion in smooth muscle cell-specific IL-4Rα-deficient mice was slightly delayed in comparison to control mice. Together, these data demonstrate that *in vivo* IL-4Rα–responsive smooth muscle cells are beneficial for *N. brasiliensis* expulsion by coordinating T_H2 cytokine responses and goblet hyperplasia, confirming previous results (Horsnell et al., 2007).

During *ex vivo* intestinal contraction studies it was found that global IL-4Rα deficiency impaired smooth muscle cell hypercontractility in response to ACh. This is in agreement with previous *ex vivo* intestinal contraction studies after IL-4/IL-13 stimulation (Akiho et al., 2005) and hypercontractility studies in STAT-6 mice (Akiho et al., 2002). Of importance, intestinal tissue from infected smooth muscle cell-specific IL-4Rα-deficient mice showed variable but attenuated contractility, showing that the absence of *in vivo* IL-4/IL-13-mediated signaling via the IL-4Rα on smooth muscle cell is sufficient to negatively influence contraction. This lead to the conclusion that worm-induced T_H2-like IL-4 and IL-13 responses are instrumental for

intestinal hypercontractility, leading to efficient worm expulsion during *N. brasiliensis* infection.

Hookworm infected humans tend to suffer from anemia resulting from the parasite clinging onto the intestine and feeding on the blood (Hotez et al., 2008). Intestine smooth muscle hypercontractility may be a mechanism activated by the host for protection from the parasite as well as for infection clearance. Increased intestinal transit previously reported following *N. brasiliensis* infection is required for host defense by means of increased worm expulsion (Collins, 1996; Vallance & Collins, 1998). This stimulated peristaltic activity is characterized by tension increases generated by intestinal smooth muscle (Vallance et al., 1997).

The increased tissue weight observed during *S. mansoni* infection may be attributed to tissue inflammation and/or eggs that got trapped in the intestine. KCl-induced tension increases correlated positively with increases in tissue weight following infection, which may be attributed to inflammation and possible altered smooth muscle structure due to egg transit. Infected global IL-4Rα-deficient mice showed the least KCl-induced tension, when compared to control and smooth muscle cell-specific IL-4Rα-deficient mice. This result demonstrated the importance of IL-4Rα in non-cholinergic-induced intestine tissue hypercontractility. KCl-induced tension increases have been previously reported following *S. mansoni*-acute infection (Moreels et al., 2001a), a result that was altered in our experiment with global IL-4Rα-deficient mice intestine tissue.

S. mansoni is a blood fluke that has female worms depositing eggs within the mesenteric veins. These eggs pierce the mucosal lining of the intestine in order to reach the lumen for excretion. Eggs that do not transit to the lumen get trapped within the intestinal wall and liver where they cause chronic inflammation characterized by type 2 granulomatous formation known to cause morbidity and mortality in both murine and human models (Weinstock & Boros, 1981).

This laboratory recently published (Marillier et al., 2010) that infected IL-4Rα$^{-/lox}$ and smooth muscle cell-specific IL-4Rα-deficient mice demonstrated similar liver and intestine egg deposit (see figure 4.1D), with intestine wall pathology resulting from egg-induced diffuse inflammation and granuloma formation similar in both infected smooth muscle cell-specific IL-4Rα-deficient mice and control mice. Smooth muscle

cell hyperplasia defined by the thickening of the muscularis propria was variable in both IL-4Rα$^{-/lox}$ and smooth muscle cell-specific IL-4Rα-deficient mice (see figure 4.1B). Smooth muscle cell-specific IL-4Rα-deficient mice also responded to egg infiltration with a type 2 immune response comparable to that of control IL-4Rα$^{-/lox}$ mice.

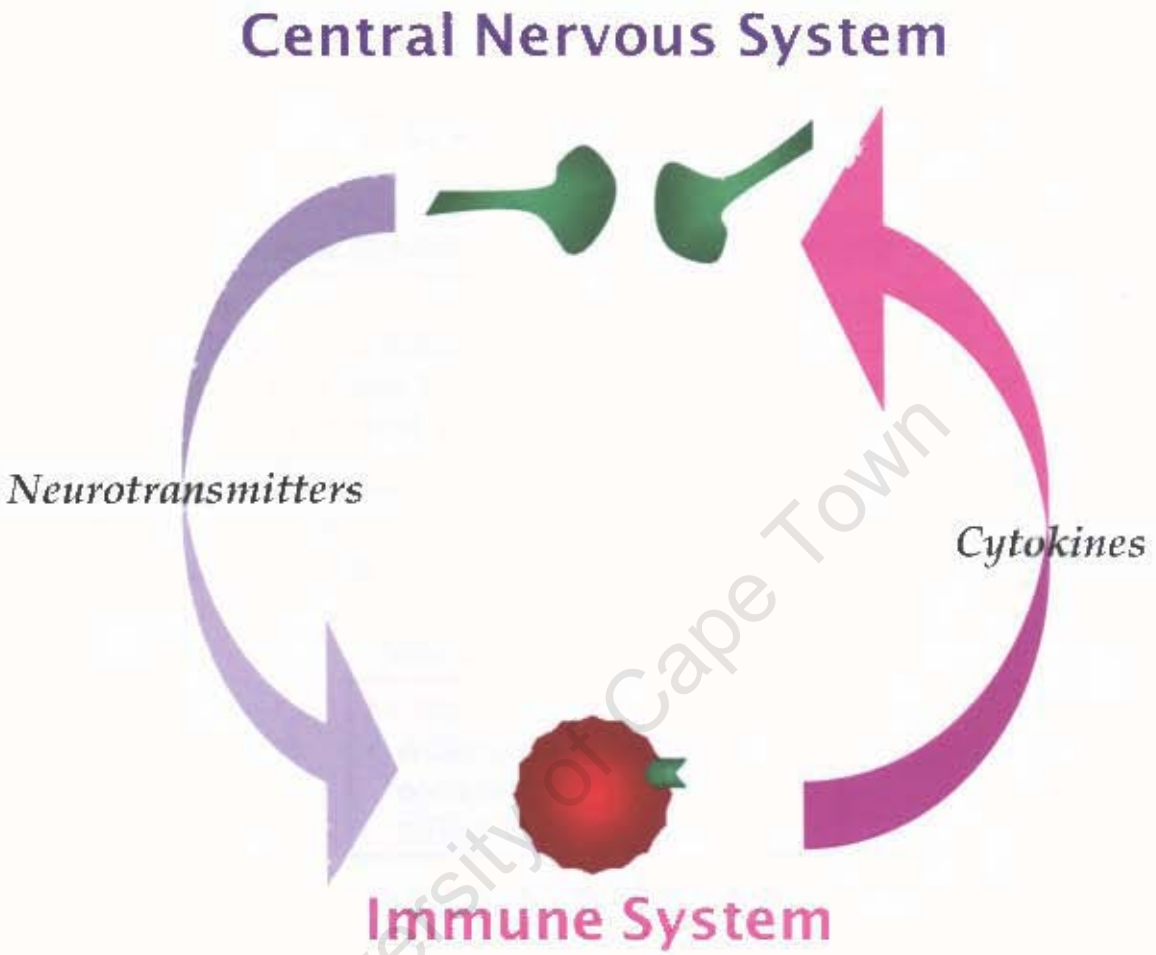
This result was contrary to that of *N. brasiliensis* infection, where smooth muscle cell-specific IL-4Rα-deficient mice demonstrated reduced type 2 immune responses compared to infected control IL-4Rα$^{-/lox}$ mice (Horsnell et al., 2007). The later results suggested that IL-4Rα-responsive smooth muscle cell either directly or indirectly influence T helper differentiation *in vivo*, discussed below in future direction. Reduced egg expulsion (see figure 4.1E) was observed in the *S. mansoni* infection in gene-deficient mouse strains with an impaired IL-4/IL-13 pathway (Fallon et al., 2000). Herbert, 2004 showed that alternatively activated macrophages are important for down-regulating intestine inflammation, which helps to prevent excessive egg retention in the intestine tissue. In the absence of alternatively activated macrophages, mice showed increased mortality caused by intestine tissue damage resulting from egg-induced host inflammatory responses and subsequent infiltration of gram-negative bacteria from the gut flora, inducing LPS-mediated septic shock (Herbert, 2004). Even so, not directly shown, retaining of eggs may have also contributed to the enhanced mortality in *S. mansoni*-infected smooth muscle cell-specific IL-4Rα deficient mice (Marillier et al., 2010).

These results may also explain motility-related GI problems (King et al., 2001) such as diarrhea in human schistosomiasis. These GI alterations also suggested that *S. mansoni* infection affects intestinal physiological responses that may be required for host protection. Intestine ileum hypercontractility has been shown in longitudinal muscle strips during chronic schistosomiasis (Moreels et al., 2001a; Moreels et al., 2004). Here, it is shown that *S. mansoni* induced an increase in cholinergic intestine hypercontractility during acute schistosomiasis in control IL-4Rα$^{-/lox}$ mice (see figure 4.4A), whereas global IL-4Rα$^{-/-}$ mice isolated intestine tissue demonstrated impaired hypercontractile responses. This result demonstrated IL-4/IL-13 mediated hypercontractility, similar to that observed following *N. brasiliensis* infection in this study (see figure 3.5).

Intestine tissue from infected smooth muscle cell-specific IL-4Rα-deficient mice was variable but not significantly increased compared to the non-infected control mice, suggesting that IL-4/IL-13-mediated hypercontractility is caused by direct smooth muscle cell stimulation via IL-4Rα signaling. The observed variability may indicate that IL-4Rα-independent factors may also contribute to observed hypercontractility in *S. mansoni*-infected mice. IL-4Rα-dependent tissue hypercontractility seems to contribute to the efficient egg transit leading to reduced intestinal histopathology, in turn preventing premature mortality in IL-4Rα$^{-/lox}$ mice.

This demonstrated that tissue hypercontractility is dependent on IL-4Rα responsiveness, with smooth muscle cells being the major contributors. The hypercontractile responsiveness was not exclusive to ACh stimulation, but to KCl stimulation as well. The similarity of these results shows that eggs borrowing through the muscle layer induce structural alteration that due to tissue hypertrophy allow KCl to induce intracellular Ca^{2+} activation and hence smooth muscle cell contractions in a manner comparable to that of ACh activity. This result is similar to what has been previously reported following chronic *S. mansoni* infection (Moreels et al., 2001amorel 2001).

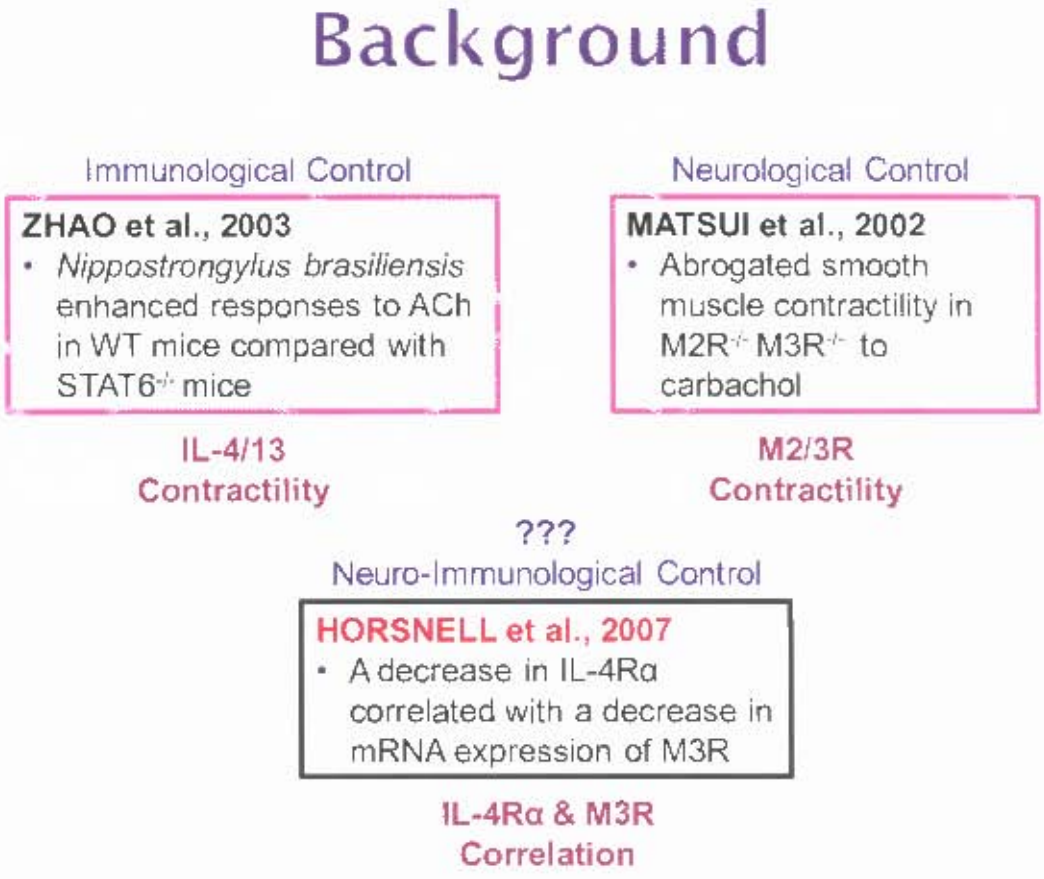
In conclusion, these results suggest that IL-4/IL-13 responsive smooth muscle cells play a role in hypercontractility by improving parasite egg migration and excretion thereby reducing intestinal driven morbidity and preventing early mortality. Further evidence is provided that the host mediates egg expulsion and that both hematopoietic and non-hematopoietic cells are required for host protection and survival.



Picture A

Schematic representation of neuro-immune interaction

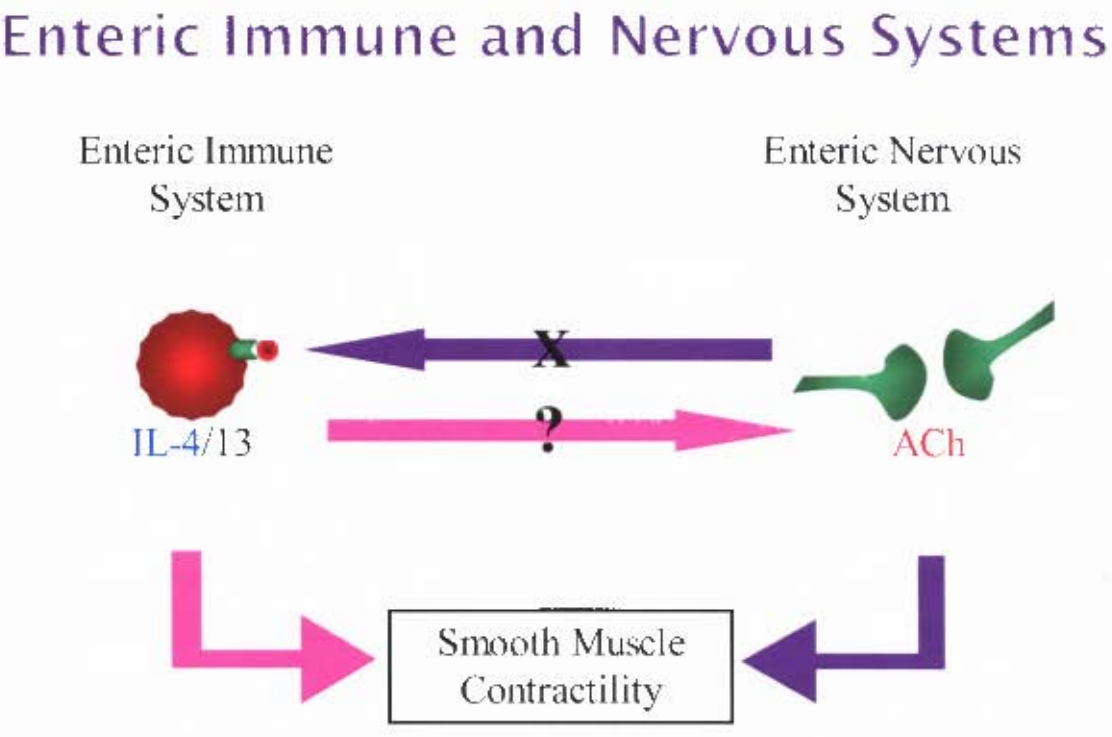
The immune and nervous system have a bi-directional communication network. Immune products signal the nervous system and the nerve system in turn modulates immune responses.



Picture B

Schematic representation of neuro-immune research summary

The immune system and the nervous system are able to control acetylcholine-induced smooth muscle hypercontractility. A possible bi-directional communication network between these two systems in acetylcholine-induced smooth muscle hypercontractility is also demonstrated.



Picture C

A schematic representation of a proposed pathway of enteric immune and nervous system influence of smooth muscle cell hypercontractility

Acetylcholine can influence smooth muscle hypercontractility in the presence of IL-4 or IL-13. IL-4 can also enhance smooth muscle hypercontractility directly without enteric nervous system influences, suggesting IL-4 effects on smooth muscle hypercontractility are independent of neuronal activity. Acetylcholine can also directly enhance smooth muscle hypercontractility without enteric immune system influences. What is currently unclear is if T_H2 cytokines can directly influence neuronal activity, leading to smooth muscle hypercontractility, and their mechanisms of action. [**X**: not required, **?**: unclear]. (Horsnell et al., 2007; Matsui et al., 2002; Zhao et al., 2003).

Future direction

It is demonstrated that IL-4Rα-responsive smooth muscle cells drive worm-induced intestine hypercontractility and influence host protective T cell responses needed for immunity to *N. brasiliensis* or *S. mansoni*, with associated changes of neurological receptors (Horsnell et al., 2007; Marillier et al., 2010). This led to a hypothesis that IL-4Rα-mediated neuro-immunological control is important for immunity to helminth infection. The aim of future studies is to dissect the role of IL-4Rα-responsive smooth muscle cells and their interrelationship with physiology, neurological innervations, and the influence on the immune system during helminth infection (i.e. *N. brasiliensis* and *S. mansoni*) on a cellular and molecular level. An important problem question to answer is; which other immunological-relevant cell sub-populations might influence smooth muscle cell contraction. Possible candidates are macrophages, dendritic cells, T and B lymphocytes, as all of them are involved in Type 2 immune responses, hence likely candidate genes. In the host laboratory cell type-specific IL-4Rα deficient mouse models for all above mentioned immunological cell types are established. It is suggested to test these mouse strains in ACh-induced contraction studies during infection with *N. brasiliensis* and *S. mansoni*, as established and shown in this work.

It is further suggested to investigate the interrelationship of smooth muscle cells, enteric neurons and lymphocytes by immunohistochemistry, isolation by laser dissection and/or FACS sorting in order answer the question; which intestine cell populations are affected in SM-MHC IL-4Rα$^{-/-}$ mice (and other important cell specific model from above) during infection with *N. brasiliensis* or *S. mansoni*. Important parameters to characterize include their cytokine responses by ELISA and/or Elispot analysis, as well as their proliferative response by BrdU incorporation and FACS analysis. Expression pattern will be analyzed by RT-PCR as a preparation for possible microarray analysis. Particular interest is focused on the identification of genes and factors involved in neurological responses, such as IL-1, IL-6 and MIP-α. These studies should uncover cellular interaction and factors involved in cross-regulation.

In order to gain information on which genes are involved in the regulation of contraction and other possible protective function by smooth muscle cells and other

cells, differential microarray analyzes from the intestine and/or isolated cell populations, including smooth muscle cells could be performed. These studies would give insights in IL-4 and IL-13-mediated pathways involved. To gain insight into the network of smooth muscle cells, neurons and lymphocytes regulating immune responses, co-culture systems could be established to characterize possible interaction of these cells. Smooth muscle cells/neuron co-cultures will allow the investigation of neuronal differentiation and survival by morphometrical analysis and staining with cell death marker such as active caspase-3. The release of neurotransmitters and the amount of vesicle proteins could be determined using ^{3}H-thymidine labeled ACh and antibodies against SNAP-25 and syntaxin. Ca^{2+}-homeostasis of both cell types could be analyzed by using the fluorescent dye fura-2 AM which measures free Ca^{2+} in the cytosol. Altered differentiation, induction of cell death, differences in neurotransmitter release or changes in the intracellular Ca^{2+} -level would hint towards an impaired neuronal transmission which could be involved in triggering the delayed response to worm infections.

To examine how wild type or IL-4Rα-deficient smooth muscle cells trigger lymphocyte-activation, smooth muscle cells could be pre-treated with interferon, and the proliferation of lymphocytes determined by using ^{3}H-thymidine. The production of cytokines could be measured by ELISA. Neurons and lymphocytes could be kept in co-culture to analyze how they influence each other. Therefore, the proliferation (^{3}H-thymidine) and the cytokine production (ELISA) of lymphocytes could be examined in the presence of neurons. Additionally, the neuronal Ca^{2+}-homeostasis will be investigated with fura-2 AM after stimulation of lymphocytes.

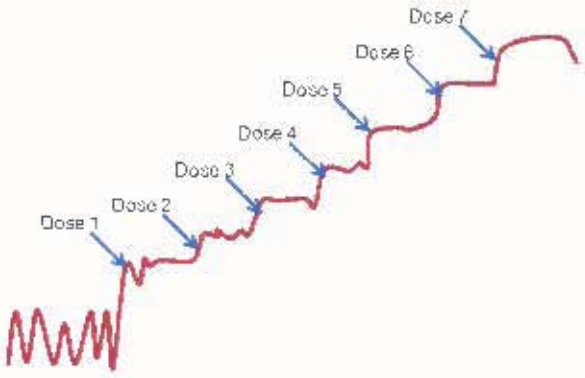
Appendix

Contractions Protocol

Cumulative method (Adapted from Tanovic et al., 2006 and modified)

1. Prepare Kreb's buffer the day of the experiment
2. Keep buffer ice cold until use
3. Turn the computer on, followed by the powerLab™, organ bath, gas, and thermostat
4. Pre-heat buffer to 37°C with few bubbles of 95:5% O_2/CO_2
5. Collect 1cm long pieces of isolated intestine tissue per mouse 10cm from the stomach pylorus into ice-cold Kreb's buffer
6. Carefully hook 1 end of the tissue to the rod and the other end to a piece of thread that hooks onto the transducer
7. Carefully lower the tissue into the buffer and hook the thread onto the transducer
8. Equilibrate tissue in Kreb's buffer for 30 minutes at 0g (0mN), i.e. do not apply tension to tissue in order to establish stabilized baseline contractile responses
9. Set powerLab™ tension readings to zero and adjust tissue tension to 9.9mN
10. Stimulate tissue with ACh, starting with the lowest concentration going up without allowing tissue to go back to resting tension between stimulations (see picture below)
11. Make sure your treatment intervals are as similar as possible (e.g. 30 seconds, etc.)

**This method is short and quick, and will give you concentration-response curves (calculated either manually or with the software)



Non-cumulative method

Single tissue/single concentration (Adapted from Goldhill et al., 1997 and modified)

1. Prepare Kreb's buffer the day of the experiment
2. Keep buffer ice cold until use
3. Turn the computer on, followed by the powerLab™, organ bath, gas, and thermostat
4. Pre-heat buffer to 37°C with few bubbles of 95:5% O_2/CO_2
5. Collect seven 1cm long pieces of isolated intestine tissue per mouse 10cm from the stomach pylorus into ice-cold Kreb's buffer
6. Carefully hook 1 end of each tissue to the rod and the other end to a piece of thread that hooks onto the transducer
7. Carefully lower each tissue into the buffer and hook the thread onto the transducer
8. Equilibrate tissues in Kreb's buffer for 30 minutes at 0g (0mN), i.e. do not apply tension to tissue in order to establish stabilized baseline contractile responses
9. Set powerLab™ tension readings to zero and adjust tissue tension to 9.9mN
10. Stimulate each piece of tissue with a single concentration of ACh, i.e. tissue in chamber #1 will be stimulated with -9 LOG [M], while the one in chamber #7 will be stimulated with -3 LOG [M]

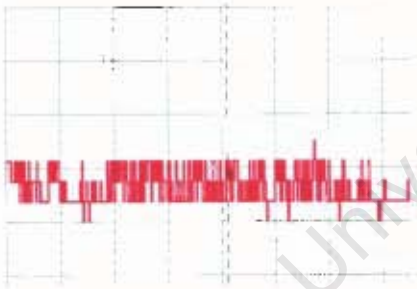
Single tissue/various concentrations (Adapted from Zhao et al., 2003 and modified)

1. Collect 1cm long pieces of intestine tissue per mouse, 10cm from the stomach pylorus
2. Weigh the tissue immediately on a petri dish
3. Carefully hook 1 end of each tissue to the rod and the other end to a piece of thread that hooks onto the transducer
4. Carefully lower each tissue into the buffer and hook the thread onto the transducer
5. Equilibrate tissues in Kreb's buffer for 30 minutes at 0g (0mN), i.e. do not apply tension to tissue in order to establish stabilized baseline contractile responses
6. Set powerLab™ tension readings to zero and adjust tissue tension to 4.95mN

7. Stimulate with KCl to check tissue viability (discard any non-responsive tissue)
8. As soon as tissue tension lowers following maximum contractile responsiveness, drain buffer (do not adjust tissue or powerLab™ readings)
9. Add fresh buffer pre-heated to 37°C making sure not to disturb tissue
10. Equilibrate tissue again for 10 minutes, re-adjust it to 4.95mN at least 2 minutes before re-stimulation
11. Repeat steps 8-10 until the last concentration

**This method is long (~1.5 hours per set)

Due to tissue handling or ice-cold buffer, some pieces may not respond to the buffer right away. So, before you discredit tissue as non-responsive (N.R.), observe it for 10 minutes in the chamber and if you don't see improvement, you can confidently term it N.R.



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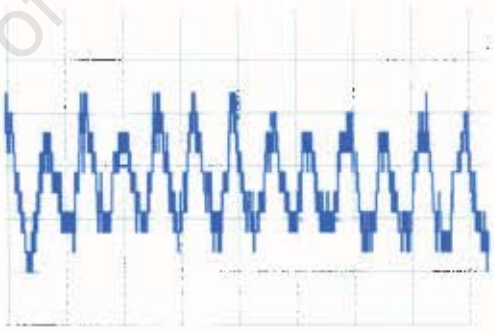

GOOD trace

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