Evaluating Vaccine Candidates for Filariasis

by

Christopher Paul Morris

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DISSERTATION AND ABSTRACT APPROVED:

DATE:

Clifford Snapper — 4/16/13
Dr. Clifford Snapper
DEPARTMENT OF PATHOLOGY
Committee Chairperson

5/23/13
Dr. Edward Mitre
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
Dissertation Advisor

5/24/2013
Dr. Stephen Davies
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
Committee Member

4/16/13
Dr. David Scott
DEPARTMENT OF MEDICINE
Committee Member

4/16/13
Dr. Thomas Nutman
National Institute of Health
Committee Member

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DEDICATION

I would like to dedicate this work to my children Caleb, Audrey, Brennan and Brielle. I hope that you will continue to find enjoyment in learning.

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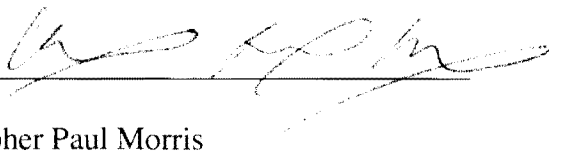
[Signature] 

Christopher Paul Morris

ABSTRACT

Evaluating Vaccine Candidates for Filariasis:

Christopher Paul Morris, Ph.D., 2013

Thesis directed by: Edward Mitre, M.D.,

Associate Professor, Department of Microbiology and Immunology

Parasites of the family Onchocercidae, commonly referred to as filarial worms, can cause severely devastating conditions such as elephantiasis and blindness. While there are programs currently in place to try to control or eradicate these diseases through mass drug administration, there are no vaccines available to prevent infection with these pathogens. There has been an interest in vaccination with "hidden" antigens, antigens to which the host does not mount an adaptive immune response during infection, in helminths. We found that the soluble fraction of the intestine (GutAg) of *L. sigmodontis* exhibits some hidden qualities, in that IgG antibody titers to these antigens are much lower than to the soluble fraction of the whole worm (LsAg), and that cytokine production by splenocytes is lower in response to GutAg than to LsAg. However, immune responses associated with allergy, including IgE titers and Basophil activation, were similar in response to LsAg and GutAg. Furthermore, vaccination with GutAg and CpG/Alum was not protective against challenge infection.

Similarly, a vaccination protocol using LsAg and CpG/alum did not protect against challenge infection. However, vaccination with irradiated larvae results in around

80% protection, yet this method of vaccination is not practical in humans. We hypothesized that comparing the immune responses in the irradiated larval vaccinated mice compared to the LsAg vaccinated mice may provide some insight into future methods of vaccination. We found that while vaccination with irradiated larvae (iL3) and LsAg with CpG/Alum induced similar IFN-γ production in response to LsAg, IL-4 production was much higher in the iL3 vaccinated mice. Vaccination with LsAg adsorbed to alum induced strong IL-4 responses to LsAg, but again did not result in a protective immune response. An analysis of humoral immune responses suggested that antibodies may play some role in protection, but vaccination of J_H mice showed that antibodies are not necessary for protection after iL3 vaccination.

Although antibodies are not necessary for protection in iL3 vaccinated mice, antibodies directed against membrane associated intestinal proteins have shown protection in many models of helminth infection. In order to identify proteins found only within the intestine of the parasite, we dissected adult female *B. malayi* into three anatomic fractions of the gut, body wall, and uterine tubes. We used a high throughput RPLC/MS-MS to determine proteins present within each anatomic fraction in order to determine transmembrane proteins within the digestive tract of *B. malayi*. Gene set enrichment analysis of our results showed a bias for transporter proteins within the gut, cytoskeletal and immunological proteins within the body wall, and transcription and nuclear regulation proteins within the uterine tubes. Analysis of gut enriched proteins showed 75 named proteins likely to be secreted or have transmembrane domains. These proteins, especially the 5 proteolytic enzymes identified to be enriched within the intestine, may prove fruitful for future vaccine research.

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CHAPTER 1: Introduction*

FILARIAL WORMS AND THEIR COMPLICATIONS

Filarial worms are parasitic helminths from the family of Onchocercidae. This family includes many parasites of humans, including: *Brugia malayi*, *Brugia timori*, *Wuchereria bancrofti*, *Mansonella perstans*, *Mansonella ozzardi*, *Mansonella streptocerca*, *Onchocerca volvulus, Loa loa*, and occasionally *Dirofilaria immitis*. While these infections are commonly asymptomatic, infections with certain species sometimes induce severely morbid conditions including elephantiasis and blindness (See table 1). In addition to direct effects on human health, members of this family parasitize other mammalian hosts which can have economic impacts on society. Furthermore, *Dirofilaria,* which only rarely causes clinical symptoms in humans, is an extremely devastating pathogen in animal companion medicine (98).

EPIDEMIOLOGY

Lymphatic filariasis is endemic in 72 countries, and an estimated 1.3 billion people are at risk of becoming infected. It is located mainly within India, Southeast Asia, Africa, South America, and the Caribbean (116). It is suspected that over 120 million people are infected with one of the causative agents of lymphatic filariasis, *W. bancrofti, B. malayi*, or *B. timori*, and that the worldwide prevalence of genital disease or lymphedema/elephantiasis is over 40 million (157). Furthermore, another 37 million people are infected with *Onchocerca volvulus*, of which, half a million are afflicted with blindness or visual impairment (6; 116).

BIOLOGY AND LIFE CYCLE

Filarial worms undergo a complex life cycle requiring both definitive and intermediate hosts. In general, infectious larvae (L3) infect their definitive host through the bite of an arthropod vector, and undergo two molts to reach the adult stage. Adults are sexually dimorphic, and thus require copulation in order to produce microfilariae (MF). The need for

Table 1: Clinical disease and complications of infection with filarial worms

Disease	Organism	Acute Symptoms	Chronic Symptoms	Notes
Lymphatic filariasis	*W. bancrofti* *B. malayi* *B. timori*	Fever, lymphangitis, lymphadenitis	Lymphedema, Elephantiasis, Hydrocele	
Onchocerciasis	*O. volvulus*	Onchodermatitis	Blindness, Keratitis, Hanging groin, Hydrocele	Risk of post-treatment eye and skin inflammation
Loiasis	*L. loa*		Urticaria, Calabar swelling, Glomerulopathy	Risk of post-treatment encephalopathy and death
Dirofilariasis	*D. immitis,* *D. repens*		Pneumonitis, Cough, Coin Lesion	Very expensive workup, increasingly recognized, possible emerging zoonosis

repeated copulation in order to continue producing offspring is variable depending on the parasite (71). MF travel either through the dermis or the circulatory system and are taken up during blood-feeding by the arthropod vector. In the intermediate host, microfilariae undergo another two molts in order to develop into infectious larvae. The hosts, localization of adults and MF and timing of molts vary depending on the species of filarial worm.

In the two genera that cause lymphatic filariasis, the intermediate host is a mosquito. *Culex, Aedes, Anopheles* and *Mansonia* species are all possible intermediate hosts (116). After the L3s are injected during blood-feeding, the L3s migrate to the lymphatic system, where they develop into the adult stage. After being released by the adult females, the MF move throughout the circulatory system until being taken up by the vector.

The life cycle of *Onchocerca volvulus*, the causative agent of onchocerciasis, while similar to other filarial parasites, has some distinct differences. This parasite is transmitted by the bite of the blackfly *S. damnosum*. After entering the dermis, larvae migrate through subcutaneous and muscular tissues. These larvae ultimately molt to adults which reside in subcutaneous nodules. Microfilariae released from these parasites travel throughout the dermis until being taken up by the vector.

The L. *sigmodontis*/BALB/c model

The model that we have chosen to work with, in our laboratory, is to infect BALB/c mice with *Litomosoides sigmodontis.* The major benefit of this model is that it is the only mouse model of filariasis wherein the parasite can conduct its entire life cycle. As a mouse model, many immunological assays can be performed, that are not available in other models. The life cycle of *Litomosoides sigmodontis*, like other filarial worms, utilizes both a mammalian definitive host, and an arthropod intermediate host. In the natural life cycle, infective larvae are

transmitted to the definitive host, the cotton rat (*Sigmodon hispidus)*, by the bite of the tropical rat mite (*Ornithonyssus bacoti*). The infective larvae migrate through the host dermis and subcutaneous tissue until contacting host lymphatic channels. The larvae enter the lymphatic channels and migrate to the host pleural cavity within the first few days PI. Within the pleural cavity, larvae molt to the L4 stage 8-10 days PI and to the adult stage 24 days PI (73). 51 days PI, cotton rats start to become microfilaremic, and MF (also known as L1 stage) can be transmitted again to the vector where they will develop to the L3 stage. The L2 stage is an intermediate stage located only within the vector. After L3 infect the mammalian host, they develop into the L4 stage 7-10 days PI, and into adults 20-28 days PI.

For our experiments, the life cycle of *L. sigmodontis* is maintained in a colony of Mongolian gerbils (AKA jirds, *Meriones unguiculatus*) and tropical rat mites. While various methods have been utilized throughout the world for experimental infection of mice, we have chosen to allow tropical rat mites harboring infective larvae to feed on jirds and perform a pleural lavage on the jird 4 days PI(66). This is an easy method of obtaining large numbers of undamaged infective larvae capable of reaching the host pleural cavity. The infective larvae can then be counted, and specific numbers of larvae can be injected subcutaneously into the nape of the neck of a mouse. In general, this allows for a 50% yield of infective larvae (66). Furthermore, almost all mice become microfilaremic, as opposed to about half of infected mice becoming microfilaremic after injection of larvae obtained by dissection of mites (66; 115).

Worm Anatomy

Nematodes are extremely complex parasites, but the adult is made up of 4 major anatomic structures. These include the body wall, the pseudocoelom, digestive tract, and reproductive tract (85).

The body wall is made up of the worm musculature, epidermis, cuticle, epicuticle, and surface coat, and is the major barrier between the parasite and its host environment. The most superficial layer of the body wall is the surface coat, which is a layer of glycoproteins analogous to a glycocalyx (85). Just deep to the surface coat is a lipid layer termed the epicuticle, which is followed by the collagenous cuticle. The cuticle is produced by an underlying syncytial layer of cells referred to as the epidermis. There are 4 outpouchings of the epidermis that contain the nuclei of these cells, termed the lateral, ventral and dorsal cords (81). Nerves that innervate the worm musculature run along the dorsal and ventral cords. Similarly, associated with the lateral cords are secretory glands and secretory ducts that release secretory products at the secretory pore (81). Between the cords, there are 4 quadrants of muscles with up to 9 myocytes per quadrant (108). These myocytes have an external contractile portion, and a deeper area associated with metabolic function and glycogen storage.

The most anterior portion of the worm digestive tract is the cuticle-lined buccal cavity, followed by the muscular pharynx (85). The majority of the length of the digestive tract is made up of the intestine of the worm which is a single layer of epithelial cells on a basement membrane. At the posterior end of the worm is the cuticle-lined anus, which has a depressor ani muscle. Food is moved into the digestive tract by the muscular pharynx, and then pushed through the intestine by a combination of high hydrostatic pressure from the pseudocoelom and opening of the anus (85).

While nematodes do not have any functioning circulatory system, their pseudocoelom is full of hemolymph which has direct interaction with most cells in the parasite. Nutrients absorbed either through the intestine or the body wall will enter the hemolymph and from there can be taken up by other cells. The hemolymph maintains a high osmotic pressure through ions

and other molecules such as the disaccharide trehalose (85). This high osmolarity is necessary for the high turgor pressure within the pseudocoelom which acts as a hydrostatic skeleton for the nematode.

The final major anatomic structure within the nematode is the reproductive tract. Filarial worms are sexually dimorphic, but our focus will be on the adult female *Brugia malayi*. The female has two ovaries at the posterior end of the worm that produce gametes (46). These gametes move anteriorly to the seminal receptacles. There they mix with semen obtained from adult males, and develop into microfilariae within the uterine tubes. The two uterine tubes merge into the vagina at the anterior end of the worm (46; 81). Fully developed microfilariae are released through the action of the muscular ovejector.

NATURAL IMMUNITY

The existence of individuals within highly endemic areas that do not harbor adult worms or develop pathological sequelae suggests the possibility of natural immunity in some humans. In humans, natural immunity is associated with increased type 1 immune responses to worm antigens (37; 123), and with a differential recognition of worm antigens compared to non-immune individuals (37). The presence of these putatively immune individuals has been corroborated with animal models. In the *O. ochengi*/cattle model, it was shown that uninfected cattle living in an endemic area were more resistant to infection than infected animals (144; 148). However, this protective effect was not complete, as even the endemic normal individuals became infected when placed in a very highly endemic area. In the *Brugia*/rhesus model, which is permissive to infection, the presence of one monkey that did not develop microfilaremia despite 20 repeated subcutaneous infections also suggests the presence of putatively immune individuals in this model as well (158).

Animal models have been used to elucidate those factors necessary to induce a protective immune response. By far, the majority of this work has been done with the *Brugia*/mouse model of filariasis. In this model, infection does not result in patency, and all worms are cleared between 6-12 weeks PI depending on the strain of mouse infected (120). While both B and T cells contribute to protection in this model, transfer studies have suggested that the T cell's role is to activate B cells which, once primed, are sufficient to induce a protective effect (111; 134). B cells, specifically B1 B cells and IgM antibodies, recruit immune effector cells and initiate granuloma formation (110; 111; 119). These granulomas, which commence 2-6 weeks PI and are made up of eosinophils, macrophages and multinucleated giant cells, have been shown to surround and kill worms (120). Other immune factors that play a role in protection in this model include the type 2 cytokines IL-4 and IL-5, the type 1 cytokine IFNγ, and IgE antibodies (16; 121; 135).

In mice infected with *L. sigmodontis*, permissiveness is dependent on the strain of mouse used. The BALB/c, BALB/k and BALB/b mice are the only mice permissible to infection, and female mice in the BALB/c model are more susceptible to infection than male mice (54; 115). The factors involved in resistance have been studied, but have not been fully elucidated at this point. The higher susceptibility of BALB/c mice compared to BALB/b mice suggests that MHC type plays some role in susceptibility, yet MHC is probably not the most important factor in resistance, since the highly resistant B10.D2 mice and the susceptible BALB/c mice both display H2D (115). Most likely, cellular immune responses play a large role in protection. The resistant C57BL/6 mice respond with both more type 1 and Type 2 cytokines in response to worm antigen, compared to BALB/c mice, and protection in C57BL/6 mice is dependent on IL-4 (10; 82).

WORM SURVIVAL IN THE HOST

Filarial worms are able to live within their host typically for many years despite host immune defenses. Their ability to accomplish such a feat is due to many factors including using methods to modulate host immune responses. Filarial worms are also known to utilize mechanisms to directly protect against host defenses.

Immunomodulation

It has been suggested that worms have derived methods of modulating host immune responses by producing factors that either inhibit specific immune pathways or stimulate desired pathways. Empiric evidence to this conclusion is a decreased ability of splenocytes of infected hosts in various models to proliferate in response to stimuli during chronic infection. Furthermore, transplantation experiments with adult *Litomosoides sigmodontis* have shown that naïve cotton rats (the natural host) will reject and kill adult worms very quickly, yet cotton rats that have been exposed to L3s or irradiated L3s will accept the transplant of adult worms (138). This suggests that there are events that occur within early infection that prime the host to tolerate the adult stage. Similarly, adult worms are necessary for microfilarial survival within the host (62).

Other evidence for the importance of suppression of host immune responses in the survival of filarial worms comes from the Maizel lab. First, infection of BALB/c mice with *L. sigmodontis* leads to an increase in levels of $CD25^+$, CTLA-4^+ and $GITR^+$ (glucocorticoid - induced TNF receptor family-related gene) T cells (141). Similarly, within 12 days of infection, there is an increase in the number of $FOXP3^+CD4^+$ cells within the pleural cavity of mice (142). More importantly it was shown that the administration of anti-CD25 antibodies in conjunction with anti-GITR or anti-CTLA-4 antibodies aided worm clearance, and that the administration of anti-CD25 (PC-61) alone may decrease worm numbers slightly.

On a molecular level, *Brugia malayi* produces a macrophage migration inhibition factor (BmMIF), prostacyclin and prostaglandin E_2, abundant larval transcripts, a transforming growth factor beta ortholog, an IL-16 ortholog, and proteins with phosphorylcholine additions (20; 89), (50). These are all used to influence the host immune system, and their effects are outlined in table 2.

Protective Mechanisms

In addition to immunomodulation, filarial worms use more direct methods to survive host defenses. Several antioxidant proteins including thioredoxin peroxidase, glutathione peroxidase, and superoxide dismutase present in the surface coat aid in defense against oxidative damage (35; 36; 122; 149; 151). The parasite also contains cystatins and serpins which, in addition to other functions, may prevent host proteolytic enzymes from digesting the worm (92; 163).

DISEASE DEVELOPMENT IN LYMPHATIC FILARIASIS

Disease development in lymphatic filariasis appears to be a complex multi-step process. The majority of infected individuals do not develop severe chronic disease, and those that do develop disease tend not to be microfilaremic. The somewhat canonical description of disease development in humans is that lymphatic dilatation occurs as a consequence of worm products, lymphatic damage occurs because of an immune response directed against dead or dying worms, and that the damaged lymphatics allows a milieu for secondary bacterial or fungal infections which eventually results in elephantiasis. Animal models provide some additional insight into the overall development of disease. Animal models that involve gross lymphedema include *Brugia* in rhesus monkeys, *Brugia* in cats, *Brugia* in ferrets, and *Brugia* in nude mice.

In cats, dilatation of lymphatics first occurs after 20 days PI when the L4 exit the lymph node and descend the afferent lymphatic vessels (140). This timeframe correlates with a major decline in live worm numbers (140), and the dilatation may be a response to the dead adult

Table 2: Immunomodulatory factors present in filariasis

Immunomodulatory factor	Action on immune system	Evidence
Macrophage migration inhibitory factor	Pleiotropic. Increases IL-1, IL-6 and TNFα	(107)
IL-16	Chemoattractant for TH1 cells and $FOXP3^+$Tregs. Initiates $FOXP3^+$ Treg differentiation. Inhibits T cell proliferation.	(5)
Abundant larval transcripts	Shown to Aid survival of *Leishmania* after transfection and increase GATA-3 and SOCS-1 transcription.	(52)
Transforming Growth Factor β	Treg and TH17 differentiation	
Phosphorylcholine	Proliferation of B1 cells. Suppression of B and T cell proliferation. TH2 skew.	(53)
JUV P120	May be bound by anti-sheath antibodies.	(154)
Complement Factor C6	Part of membrane attack complex. Role in parasite is unclear.	

worms. After infection into the foot, most cats develop an enlarged popliteal lymph node within weeks of infection which is followed by development of swollen, varicosed afferent lymphatic vessels (41). Over time, lymph nodes and vessels become replaced by fibrous tissue and lymphatic channels form in response to occlusion of the original channels (125). Despite changes in lymphatic vessels, only a minority of cats develop frank lymphedema of infected limbs (124).

Information from both the *Brugia* in the ferret model and *Brugia* in the rhesus model suggest a correlation between an immune response that prevents microfilaremia and disease development. In infected rhesus monkeys, it has been observed that monkeys that develop lymphedema develop relatively strong immune responses to filarial antigens and typically are not microfilaremic (51). Finally, ferrets, which only develop a transiently patent infection, develop a temporary lymphedema during an initial infection but will also develop chronic leg edema upon repeated infections (33; 60). Some nude mice infected with *Brugia malayi* will develop an elephantoid syndrome (152), which may be due to a lack of regulatory T cells and an overzealous innate immune response.

In humans that develop disease, it is thought that, similar to cats, lymphatic vessels become non-functional because of both hyperplasia and dilatation of lymphatic vessels. Lymphatic changes are a result of innate immune responses that develop against dying worms and their endosymbiont *Wolbachia* (116). Some evidence for this is that individuals who do not develop a disease state produce more IL-10, TGFβ, and IgG4 in response to worm antigen (116), whereas the diseased state is associated with high levels of IFNγ, IL-5, IL-6, IL-8 and IL-13 (116). While this cytokine profile is associated with an adaptive immune response, the development of disease in nude mice suggests innate immune responses play a major role. One

result of some innate responses is the production of vesicular endothelial growth factors which may lead to endothelial hyperplasia and lymphangiectasia (116). This state results in impairment of lymphatic flow, and extravasation of fluid. Fluid accumulation within the more distal areas of the limbs and a lack of proper filtration provide a milieu for both bacterial and fungal growth. The resulting repeated bacterial and fungal infections, and infiltration of plasma cells, macrophages, and eosinophils result in fibrosis of the dermis and hyperplasia of connective tissues (70; 116).

TREATMENT

Chemotherapeutic treatment generally includes the use of a 1 day treatment of albendazole in combination with either diethylcarbamazine or ivermectin (63). This treatment strategy has been shown to decrease microfilaremia for up to a year, and is beneficial in blocking transmission (116). However, this regimen is not macrofilaricidal, meaning that this does not kill adult worms, and therefore requires yearly administration in order to prevent transmission (63). Furthermore, this treatment strategy does not reverse the chronic pathological symptoms after they have developed.

Treatment of individuals who have already developed clinical sequelae of infection focuses on preventing superinfection and promotion of lymphatic drainage in afflicted areas. Promotion of lymphatic drainage can be accomplished by exercising, wrapping with ACE bandages, and raising afflicted limbs (24). In some cases, such as hydrocele, surgery can be beneficial in reversing symptoms.

CONTROL PROGRAMS

Currently the Global Program to Eliminate Lymphatic Filariasis, the Onchocerciasis Elimination Program of the Americas, and the African Programme for Onchocerciasis control, all utilize repeated mass drug administration (MDA) of anthelmintics in an attempt to prevent

transmission of filarial worms. These regimens are not effective at treating ongoing infection, but instead prevent transmission of disease by decreasing microfilaremia in infected individuals. While the duration that MDA is required to eradicate the disease from an area is still debated, a period of 6-10 years has been suggested to allow existing adults to die off (63; 116).

VACCINES

Utility of a Vaccine

The rationale for spending the time, energy, and money to develop vaccines against filarial diseases when the world is already investing heavily in MDA programs to disrupt filariasis transmission was recently outlined by Babayan and colleagues (11). While ongoing MDA programs are tremendously beneficial, issues of incomplete MDA coverage, inability to provide MDA in *Loa*-endemic regions, long duration of MDA required, population shifts, and the potential for development of drug resistance during MDA all limit the likelihood that current approaches will result in complete eradication of filarial diseases. Thus, a vaccine would be a useful tool in the eradication campaign.

Potential Problems with Vaccination

Any vaccination protocol against lymphatic filariasis carries with it two major risks. First, disease development in lymphatic filariasis appears to be partially immune mediated, and it is possible that a vaccine could be effective at decreasing worm burden and simultaneously exacerbate pathology. The *Brugia* in ferret model clearly demonstrates this. Ferrets were vaccinated with both live and dead MF intradermally and intravenously. After challenge infection, these ferrets exhibited lower adult and MF parasite burdens and higher pathological sequelae compared to control animals (33). For this reason, any vaccine candidate found will need to be examined in a suitable animal model of filariasis that exhibits relevant disease state to humans. The other potential problem with vaccination is the possibility of previously exposed individuals having allergic reactions to components in the vaccine. Because of the type 2

Table 3: Treatment for filariasis

Drug	Drug Class	Effective against	Mechanism of Action	Concerns
Albendazole	Benzimidazole	*W. bancrofti* *B. malayi* *L. loa* *O. volvulus*	Prevent microtubule formation	
Ivermectin	Macrocyclic lactone	*B. malayi*	Bind Glutamate gated chloride channels, prevents release of GABA, prevent pharyngeal muscular contractions and ES apparatus in Brugia MF (Moreno 2010)	
		B. timori		
		O. volvulus		Requires administration of prednisone if MF are found in the eyes
		M. streptocerca		
		D. immitis (in dogs)		
		L. loa		
Diethylcarbamazine	Piperazine Derivative	*O. volvulus*	Associated with arachidonic acid pathway and requires iNOS	Dermatitis in response to rapid death of MF in the skin
		M. streptocerca		
		B. malayi		
		B. timori		
		L. loa		Can cause fatal encephalitis
		W. bancrofti		
		D. immitis (in dogs)		
Doxycycline		*Wolbachia*		Cannot give during pregnancy or to pediatric population
Melarsomine	Arsenical Compound	*D. immitis* (in dogs)		Thromboembolism (can be fatal)

responses that infected individuals develop to helminths, most exposed individuals develop IgE antibodies toward worm antigens (44; 95). The presence of IgE antibodies can then induce type 1 hypersensitivities upon exposure to worm antigens. This is not just a theoretical problem; this specific phenomenon occurred during human safety trials of a hookworm vaccine *Necator americanus Ancylostoma* secreted protein 2 (Na-ASP-2) (44). Individuals who had pre-existing IgE antibodies to Na-ASP-2 developed urticaria upon vaccination with Na-ASP-2.

Requirements in a vaccine

In order for a vaccine to be useful, it would need to be both safe and effective. In order for a vaccine to be safe, it cannot induce allergic responses in exposed individuals, induce autoimmune disease in humans, or exacerbate pathological sequelae after infection. There are, however, several different ways that a vaccine could be effective. A vaccine could be effective at protecting the individual from infection, preventing transmission, or preventing pathological sequelae from developing even without protecting from infection.

Previous research examining the induction of a protective immune response with vaccines or repeat infections

Strategies of inducing a protective immune response, including trials of vaccines, repeat infections, and concomitant immunity have been carried out in at least 27 different models of filariasis, and therefore the previous work in this field is somewhat hard to interpret. The most important factor perhaps in determining the relevance of each of these models is the extent to which the model is permissive for infection. For example, a large amount of work has been carried out determining efficacy of vaccines using mice infected with *Brugia malayi* or *Brugia pahangi*. However, immunocompetent mice infected with either of these species clear these infections very quickly and never develop patent infections. In this situation, vaccine trials are used to determine whether or not clearance of worms is accelerated. This is very different than a human infection where filarial worms can survive a decade. The factors that accelerate clearance

in a non-permissive host are not necessarily the same factors that would produce an effective immune response in a host that does not typically develop protective immunity. For this reason, this section will be limited to a discussion on vaccine and repeat infection trials in permissive models of filariasis.

Concomitant immunity

Concomitant immunity (CI) is a type of immunity described in the literature, wherein an individual can harbor adult worms and MF, but display some resistance to infective larvae. For example, cats infected over 20 times with L3s will remain infected with adult worms, but no L3s will survive to adulthood during further infections. Understanding the mechanisms underpinning such a state would provide great benefit and a basis for vaccine development. However, the evidence for such an immune state remains imperfect. In humans, such a state is hypothesized because it has been shown that worm numbers plateau at around the age of 20 and then slowly decline throughout a person's life, despite the continued exposure to infective larvae. While this may be the result of a developed immune response, other factors, such as resource competition, may be at play. Of the seven models that have specifically tested for some type of CI, CI has been exhibited in very different ways. In the *A. viteae* models, Jirds develop a robust concomitant immunity very quickly (17; 118), whereas hamsters only exhibit concomitant immunity after many low dose infections (104). In the *Brugia pahangi* models, cats develop some concomitant immunity after 12 repeated infections, and further infections will not increase worm numbers after 20 repeated infections(42). Jirds however, will continue to accrue higher parasite burdens even when infected with *B. pahangi* up to 20 times , and there are contradictory reports on whether or not they develop concomitant immunity at all (43; 75; 76; 78). For *L. sigmodontis*, this has only been sufficiently studied in jirds which show stunting and decreased survival in superinfecting worms. While CI may be useful in understanding the induction of a

protective immune response, it has not yet been studied in mice infected with *L. sigmodontis*. For this reason, there is a paucity of information on how CI develops and whether this method can be used as a road-map of future research.

Protection after Chemically Abbreviated Infection (CAI)

Protection after chemical abbreviation of an infection appears not to occur for LF in humans, but does occur to some extent in many animal models of filariasis. This may be from an inability of humans to mount a protective immune response after CAI, or it may be possibly from the length of time that a person is infected prior to anthelmintic treatment. Indeed, in the *B. pahangi/*jird model, it was shown that the length of infection prior to anthelmintic treatment greatly altered protection. Jirds treated with anthelmintics 7-9 weeks PI had a 77% lower worm burden after a later challenge infection, but jirds treated with anthelmintics after patency displayed no protection after subsequent challenge(64). One very promising avenue for future research is that both dogs and ferrets show marked protection after a CAI with *D. immitis* (21; 56; 162). This approach has not yet been explored in mice infected with *L. sigmodontis*, and the mechanisms underpinning this protective response have yet to be elucidated.

Protection after vaccination

Vaccination with adult soluble antigens

Soluble antigens from adult worms have been tried in various models of filariasis. Protection from this type of vaccine approach ranges from 0-50%, with the least permissive models of filariasis garnering the best protection from soluble adult antigens. The utility of each model to screen for vaccine candidates is dependent on its ability to positively identify useful antigens. For this reason, models that show protection after vaccination with crude fractions of adult worm antigens would not be ideal for testing vaccine candidates. Therefore vaccination with soluble adult antigens provides some insight into the utility of any model for future vaccine

research. This approach has yet to be tried for the *L. sigmodontis*/BALB/c model of filariasis, and would increase the utility of this model.

Vaccination with irradiated larvae

One very effective method of vaccination in most models of filariasis is vaccination with irradiated larvae (84; 143; 155; 158; 160). While many different vaccination and irradiation protocols exist, the irradiation dose that tends to provide the best protection is one that prevents worms from developing to sexual maturity but still allows worms to survive for some time within the host (109). As long as the worms are given an adequate dose of irradiation (typically in the area of 40kRad) the host does not develop pathological sequelae from the vaccine alone (109). Despite this, vaccination with irradiated larvae is not practical in humans regardless of any safety issues. Due to the complex life cycle of filarial worms, it simply is not feasible to obtain enough parasites to vaccinate humans. Instead vaccination with irradiated larvae can be used to determine aspects of an effective immune response against filarial worms in the hopes of emulating those findings with a more feasible vaccine approach.

While this vaccine approach has shown protection in 16 different models of filariasis, the mechanism of protection appears to be different in these different models. In the *L. sigmodontis*/BALB/c model of filariasis, worm numbers are decreased as early as 3 days PI, and both antibodies and IL-5 are necessary for protection (83; 84; 96; 97). Since the worms that make it to the pleural cavity in this model survive at a similar rate in vaccinated animals as non-vaccinated animals, it has been suggested that protective responses specifically target the L3 stage of the worm and perhaps prevent localization to the pleural cavity. However, in the jird models, it has been shown that protection against *B. malayi* and *B. pahangi* after vaccination with irradiated larvae remains high whether the challenge worms are inoculated IP or SC (32; 160). The significance of this is that *Brugia* inoculated IP into a jird do not migrate, and thus

prevention of migration is clearly not the mechanism of protection in this model. Different timeframes of worm clearance post challenge in iL3 vaccinated hosts also suggests the presence of multiple mechanisms of protection (22; 84).

Histological analyses after challenge infection in vaccinated jirds show that L3s become trapped in eosinophilic granulomas (22). Furthermore, in the same model it has been shown that jirds produce antibodies after vaccination that aid in cytoadherence (91). Throughout essentially every model that has been tested, there is a differential antibody response to worm antigens in vaccinated compared to infected animals (47; 48; 86; 91; 101). However, in many of these studies, the size of differentially recognized proteins is all that is known, and the protein sizes appear not to correlate from model to model. If antibodies are truly necessary for protection, determining the identity of any of the proteins recognized by protected vs. non-protected animals could be an important starting place for finding future vaccine candidates.

In order to determine differences in cytokine responses after vaccination, some work has been done in the *L. sigmodontis*/BALB/c model of filariasis. In one study, cytokine production by splenocytes of vaccinated animals was compared to naive animals, and later vaccinated/infected responses were compared to simply infected animals (84). In these studies, vaccinated animals produced more IL-5 and IL-6 in response to worm antigen than did naive animals. Similar results were obtained within the first 2 days of infection (84).

Similarly, in our laboratory, it was shown that splenocytes from vaccinated mice produced both more IL-4 and IFNγ in response to worm antigen than did splenocytes from naïve mice (67). However, the differences between an effective immune response after vaccination and the immune response in naive mice are of little use in understanding of the development of an effective vaccine. Instead, an in-depth analysis of the immune response of protected mice as

it compares to the immune response of mice that are sensitized to worm antigens but not protected, may provide more insight into future vaccine research.

While in the first study there was some work done studying the differences between vaccinated/infected animals compared to infected animals, it is still not clear if the infected group is not protected against future infection, especially given the time frame of worm clearance in vaccinated mice. Repeat infections and concomitant immunity studies have shown a very large variation in protection after infection from model to model (76; 77; 118), and this type of research has not been carried out in the *L. sigmodontis*/mouse model of filariasis. Therefore, it is possible that the infected group may show similar levels of protection to the vaccinated/infected group were they reinfected at that time-point. For this reason, the infected group may not be suitable to use as a non-protected group of mice.

Vaccination with Hidden Antigens

One method that could perhaps overcome the issue of allergic reaction to the vaccine is to vaccinate with "hidden" antigens. Hidden antigens are antigens to which the host does not mount an appreciable adaptive immune response during infection. There is some evidence in nematodes that during normal infection the host may not typically interact with the worm intestinal antigens (99; 132). Sheep infected with *Haemonchus contortus* (a parasitic nematode of the gut in sheep) do not produce antibodies against many of the membrane associated intestinal antigens of this parasite (132). However, vaccination with these antigens, more specifically the proteolytic enzymes, induces the production of antibodies in sheep that inhibit these enzymes and protect against infection (25; 45). Similar protective results have been shown by vaccinating with specific proteolytic enzymes in various other helminth infections including Schistosomiasis, *Fasciola hepatica, A. caninum,* and *N. americanus* (4; 18; 27; 28; 30; 38; 90; 94; 112; 113; 165).

In the field of filariasis, the use of intestinal antigens as a vaccine candidate has only been tried against *Dirofilaria immitis* (99). In this study, the intestinal antigens were fractionated based on levels of solubility, and used to vaccinate mice which were later implanted with diffusion chambers containing infective larvae. The mice that were vaccinated with the soluble fraction in this study eliminated more larvae during their "infection" than non-vaccinated mice, or mice vaccinated with non-soluble fractions. In this study, the soluble, but not the insoluble, fractions were protective, which suggests that the targeted antigen may have been secreted into the intestine as opposed to being membrane bound. This was internally consistent with the finding that heavily infected dogs do produce low level antibody responses to the soluble fraction, but not the non-soluble fraction, of the intestine after long term infection (99).

MF Vaccines

There have been many attempts to immunize various hosts against the MF stage of filarial worms. There are many parasite host combinations that result in a rather short microfilaremic stage, and the end of patency is associated with the production of antibodies directed against the microfilarial sheath. This suggests that the MF stage would be an easy target for vaccine work in order to prevent disease transmission. The utilization of MF vaccines has been effective in 10 different models of filariasis; however the majority of the models that have attempted this approach have only been transiently permissive to infection. Despite this, in 2 natural host-parasite interactions (*O. lienalis* in cattle and *L. sigmodontis* in cotton rats) vaccination with MF prior to challenge infection was shown to drastically reduce patency following infection (57; 147).

While this approach is promising, there are many health concerns with this type of vaccine that would need to be addressed. First, in many models, the animals that are protected from microfilaremia trap the MF within blood vessels of the lungs, and cellular infiltration is

apparent in the lungs (34; 57; 60; 131). This results in reactive tissue eosinophilia and asthmatic changes, which strongly mimic the human condition of tropical pulmonary eosinophilia. Second, as mentioned previously, humans, rhesus monkeys, and ferrets that control microfilaremia are more likely to develop pathological sequelae in lymphatic filariasis. Finally, while the vaccination of ferrets with MF from *Brugia malayi* results in protection against MF on subsequent challenge with infective larvae, vaccinated ferrets develop more severe pathological changes compared to primary infected animals (33). For these reasons, we chose not to pursue a vaccine approach utilizing MF as a vaccine candidate.

Vaccination with individual antigens

There are many individual antigens that have shown protection in various models of filariasis, yet none of these approaches have led to sterilizing immunity (29; 36; 52; 55; 117; 130; 146; 149-151). While the function of some single antigens with protective effects are unknown, most fall into the categories of muscular proteins, proteins involved in protection from oxidation, immune altering proteins, or cuticle remodeling proteins. Many of these antigens have been found by screening sera from individuals that live in endemic areas, but are protected from infection. Protection garnered from these individual antigens is promising, yet none of these approaches has resulted in sterilizing immunity. Because of the risk of allergic reactions to this approach, we are instead focusing our research on "hidden" antigens and gaining better understanding of the factors necessary for vaccine induced protective immune responses.

Focus of our vaccine research

The exhibition of allergic responses to the hookworm vaccine in sensitized individuals made it clear that any vaccine research against a helminth infection needs to account for the possibility of allergy to worm antigens. For this reason, we have chosen to make vaccination with "hidden antigens" a priority for vaccine research. Previous studies with various helminths

have suggested that intestinal antigens may be prime candidates for hidden antigen vaccines. We have chosen to evaluate the utility of intestinal antigen vaccines both by vaccinating with the soluble fraction of intestinal antigens and by performing proteomics work to identify individual antigens of interest.

Additionally, we also wanted to gain a better understanding of the correlates of immunity in filariasis. Vaccination with iL3 has a high potential for providing insight into the correlates of immunity, and the method of protection following iL3 vaccination has yet to be fully elucidated. For these reasons, we have chosen to spend time examining the immunological differences in protective and non-protective vaccine approaches in the *L.* sigmodontis/BALB/c model of filariasis. Lastly, we have focused research on increasing the utility of the *L. sigmodontis*/BALB/c model by determining protection after LsAg vaccination and repeat infection.

GENOMICS AND PROTEOMICS WORK TO DATE

A strong understanding of parasite biology is important in the design of an anti-filarial vaccine. A draft genome of *B. malayi* was published in 2007. In the draft genome, several different aspects of the worm biology were labeled as likely anti filarial targets including molting, nuclear receptors, collagens, collagen processing, and neuronal signaling (50). It was also suggested that *B. malayi* lacked many of the enzymes involved in purine synthesis, heme biosynthesis, and riboflavin biosynthesis, and that perhaps the acquisition of these molecules from the host could be targeted (50).

The most inclusive proteomics work to date has been performed by Bennuru and colleagues. A study of the proteins present in the excretory/secretory (ES) products of various life cycle stages found 852 proteins, including 274 proteins that were previously considered predicted proteins. Interestingly, the majority of proteins found in the ES products were specific

to the parasite's stage of life. In this study, a high level of glycolytic enzymes, and immunomodulatory proteins, such as MIF and leucyl aminopeptidase (an ES-62 ortholog) were found within the ES products of adult female worms (20). Similarly, work done with the somatic antigens of *B. malayi* identified 7,103 proteins between the different stages of the parasite (19).

Proteomic gender differences between worms.

Several studies have looked at the differences in proteins of adult worms based on gender. In one such study, Jiang and colleagues identified proteins in LC-FTMS/MS after determining a gender differential with 2D DIGE (2 dimensional difference in gel electrophoresis). They identified 62 different proteins with a differential gender expression. The most interesting part of this study, however, was a follow up confirmation of the location of the production of many of these proteins by in situ hybridization (69). The benefit of this is that, although not perfect, this allows for a level of validation of data within any future organ specific proteomics research. Furthermore, when Bennuru and colleagues identified proteins present within the MF obtained from the Uterine Tube, this allows for a method to determine consistency of data for future organ specific proteome research (19).

Proteomics of the nematode gut, a source of hidden antigens

While there has yet not been done a proteomic analysis of the digestive tract of filarial worms, a study done in 2008 compared the intestinal transcriptomes of 3 different nematodes, namely *Ascaris suum, Haemonchus contortus,* and, *Caenorhabditis elegans* (161). In this study, it was suggested that the intestinal transcriptomes of nematodes were very diverse, but that there was also a core of protein families that were highly conserved between these three species. Furthermore, there was a greater conservation of expressed intestinal genes compared to genes expressed in the gonads. The conserved intestinal genes had various cellular functions and included proteolytic enzymes and oxidoreductases (161).

HYPOTHESES AND SPECIFIC AIMS

We hypothesized that since the digestive tract of parasitic filarial nematodes is sequestered within the worm, that it is likely that this organ contains antigens that would not elicit allergic reactions in previously exposed hosts. Furthermore, given the efficacy of vaccines comprised of these antigens in other nematode models, we hypothesized that a vaccine comprised of filarial intestinal antigens would induce a protective immune response in the permissive *L sigmodontis*/BALB/c model of filariasis. Even if the soluble fraction of the intestines is not protective, it is likely that the filarial digestive tract contains antigens that could be protective given individually with the right vaccination strategy. Finally, we hypothesized that there are aspects of the immune system necessary for protection after vaccination with irradiated larvae, and further study into this vaccine strategy will aid in developing a more feasible vaccine strategy.

In order to test these hypotheses, we developed the following specific aims for this project.

Specific Aim 1: Determine the safety and efficacy of a vaccine comprised of soluble intestinal antigens in the *L. sigmodontis*/BALB/c model of filariasis

Specific Aim 2: Determine differences in, and evaluate importance of, humoral and cellular immune responses in a protective compared to a non-protective immune response

Specific Aim 3: Identify potential intestinal vaccine targets in the human pathogen *B. malayi*

*Note: Some portions of the introduction are also present within the review paper "A comprehensive, models-based review of vaccine and repeat infection trials in filariasis" that is currently in revision at the journal Clinical Microbiology Reviews.

CHAPTER 2: Vaccination with intestinal tract antigens does not induce protective immunity in a permissive model of filariasis

INTRODUCTION[1]

Filarial nematodes cause diseases such as Onchocerciasis ("river blindness") and lymphatic filariasis (lymphedema and elephentiasis), debilitating conditions that have severe economic, psychological, and social impacts on affected individuals (13-15). Similarly, zoonotic nematodes such as *Dirofilaria immitis* and *Haemonchus contortus* cause severe pathological sequelae in pets and livestock (49; 98). While there are medications to treat these infections, there are currently no vaccines to prevent them. Filarial vaccines could provide a cost effective tool for aiding in the control of these diseases.

Development of vaccines against parasitic filarial worms is complicated by two major issues. The first challenge is the lack of natural sterilizing immunity. In susceptible hosts, the immune responses that develop during infection are generally inadequate to clear the parasite. Indeed, filarial worms often survive for years within permissive hosts despite the development of robust anti-filarial immune responses (98). Furthermore, in many models, these immune responses provide little or no protection against future infection (42; 43; 77; 78).

The second major challenge to filaria vaccine development is the risk that vaccination would elicit allergic responses in exposed individuals. Like other helminths, filariae typically induce a type 2 shifted immune response with production of high levels of parasite specific IgE (44; 95). Thus, a vaccine comprised of parasite antigen has the

[1] GutAg- Soluble fraction of antigens obtained from the digestive tract of adult *L. sigmodontis*
LsAg- Soluble fraction of antigens obtained from adult *L. sigmodontis*
PI-Post Infection

potential to induce allergic reactions in individuals that are currently or have previously been infected with a filarial worm (44).

The use of "hidden" antigens, antigens which are not typically targeted by the immune system during the normal course of infection, could potentially overcome both of these obstacles. Interestingly, prior studies have suggested that intestinal tracts of adult helminths may contain exactly such antigens. This concept has been best demonstrated with *Haemonchus contortus*, a nematode infection of sheep. Although infection elicits relatively low antibody levels to the gut of *H. contortus*, vaccination with intestinal antigens induces substantial protection against challenge infection through the production of antibodies against parasite intestinal tracts (25; 132). Antibodies from vaccinated animals disrupt the parasite's hemoglobinase activity, suggesting that protection occurs by inhibiting parasite metabolic activity (45).

In the field of filariasis, this approach has only been tried using a mouse model of *D. immitis* infection. Although dogs infected with *D. immitis* were shown to produce only low level antibody responses to *D. immitis* intestinal antigens, presumably because these antigens are sequestered within the worm(99), vaccination of mice with *D. immitis* intestinal antigens induced high titer antibody responses to these antigens and resulted in increased protection against challenge infection (99). While exciting, this study was limited by the use of *D. immitis* challenge into mice. *D. immitis* survives for only a short period of time in mice and has never been shown to complete its life cycle in this host (1; 166). For this reason, it is not clear if the protection garnered through vaccination was simply the result of an accelerated immune response to *D. immitis*, or if a truly novel protective immune response was produced through vaccination.

The purpose of this study was to investigate the vaccine potential of filarial intestinal antigens using the permissive *Litomosoides sigmodontis* BALB/c model of infection, the only murine model of filariasis in which infective-stage L3 larvae develop into adults that release microfilariae in fully immunocompetent mice (61). *L. sigmodontis* is a good model for investigating vaccine approaches for filariasis because the immune responses which develop in infected mice closely mimic those observed in humans (10; 61; 141). Additionally, enumeration of adult worms is reliably complete as over 95% of the adult worms can be recovered from the pleural cavity.

Specific goals of this study were to: 1) determine the extent to which intestinal antigens of *L. sigmodontis* (GutAg) are immunologically "hidden" during infection of mice, and 2) to test whether vaccination with GutAg confers protection against challenge infection.

MATERIALS AND METHODS

Infection with *L. sigmodontis*

Infectious larvae of *L. sigmodontis* were obtained by performing a pleural lavage on jirds 4 days after exposure to mites harboring infectious stage larvae as previously described (66). Briefly, Jirds were euthanized with CO_2, the peritoneal cavity was opened, and a small incision (0.5 cm) made on the ventral aspect of the diaphragm. A transfer pipette was used to flush the pleural cavity repeatedly with 10 ml warmed (37°C) RPMI (Mediatech Inc. Manassas, VA). L3 larvae were then counted using a dissecting microscope, aspirated with a 20 µl micropipettor, and placed into a syringe. 40 L3 in 100 µl of RPMI were injected subcutaneously with a 22 gauge needle into the nape of the neck of 5-8 week old female BALB/c mice.

Production of soluble antigen extracts from whole worms (LsAg) and worm intestines (GutAg)

Adult female worms were obtained from the pleural and peritoneal cavities of infected jirds. These were separated and maintained in worm culture media until dissection, up to four days using 12 well culture plates (5 worms in 3ml/well). Worm culture medium consisted of RPMI (Mediatech Cat 15-040-CV), 1% glucose (Sigma-Aldrich Co. St. Louis, MO), 100 I.U/ml Penicillin (Mediatech inc.), 100 μg/ml streptomycin (Mediatech inc), and 25mM HEPES (Mediatech, Inc)).

Worm dissections were carried out in PBS in a Petri dish using fine tipped forceps and a dissecting microscope. Two sets of forceps were used to grasp the cuticle. Pressure was applied in opposing directions causing the cuticle to tear. The cuticle was then slowly peeled away from the internal structures. The intestinal tract was then separated from the two uterine tubes and placed in ice-cold PBS. The intestines were stored at -20 °C. Soluble gut Ag (GutAg) and soluble antigen from adult worms (LsAg) were prepared by placing purified intestinal tracts or whole worms (male and female) into "D" tubes from BioPulverizer System 1 (MP Biomedicals Cat 6750-200) and run on a FastPrep-24 system (MP Biomedicals) at 4.0M/s^2 for 20 seconds. After mechanical homogenization, samples were centrifuged at 16,100 *g* for 1 minute, supernatant saved, and the pellet resuspended in another 1 ml PBS. The fast-prep run, centrifugation, and removal of supernatant was repeated on the resuspended pellet. The saved supernatants were then combined, centrifuged at 15,000 *g* for 5 minutes, and the final supernatant retrieved and saved as either GutAg or LsAg. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific Rockford, IL).

Timing of Immune Responses to GutAg and LsAg in Infected Mice

As BALB/c mice clear adult worms by 20 weeks post infection (PI), immune responses were evaluated at 8 and 22 weeks post infection (PI) to determine antigen

specific immune responses that develop in currently infected and previously infected animals. After CO_2 euthanasia, blood was obtained from mice by cardiac puncture, and plasma was obtained by separation with a plasma separator tube (BD Cat#365958).

Measurement of GutAg- and LsAg-specific total IgG, IgG1, IgG2a, and IgE antibodies

Prior to running IgE ELISAs, IgG antibodies were first adsorbed from the plasma by overnight incubation with GammaBind Plus Sepharose beads (GE Healthcare). Parasite specific antibody titers were measured by ELISA in 96 well half area EIA/RIA plates (Corning#3690). Wells were coated overnight at 4°C with 50 µl of 2 µg/ml of either LsAg or GutAg in PBS. Wells were washed in washing buffer (PBS with 0.025% Tween 20 (Acros Organics)) using an ELX 405 microplate washer (BioTek Instruments Winooski VT) and subsequently blocked with 200 µl 5% BSA (Sigma-Aldrich), 0.05% Tween 20 (Acros Organics) in PBS at 37°C for two hours and washed. 50 µl of serially diluted plasma was added and incubated overnight at 4°C. 50 µl detection antibody (purified goat anti mouse IgG conjugated to alkaline phosphatase (Mabtech Cincinnati, OH CAT3310-04)) diluted 1:1000, 2 µg/ml biotinylated rat anti-mouse IgG1 (clone A85-1 BD Cat 50331), 2 µg/ml biotinylated rat anti mouse IgG2a (clone R19-15 BD Cat 550332) , or 2 µg/ml biotinylated rat anti mouse IgE (Clone R35-118 BD cat 553419) was added and incubated 1 hour at 37°c. 50 µl Streptavidin conjugated alkaline phosphatase (Jackson Immunoresearch #016-050-084) diluted 1:1000 was then added for IgE, IgG1 and IgG2a ELISAS and incubated 1 hour at 37°c. 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich N9389) was then added at a concentration of 1mg/ml in sodium carbonate buffer (KD Medical Cat CUS-0242) and incubated for 35

minutes at room temperature. OD values were read at 405nm with a Victor^3 microplate reader (Perkin Elmer). OD values of plasma dilutions were graphed using graphpad, and a four parameter curve was calculated for each mouse. This curve was used to determine the dilution of plasma from each mouse that would provide an OD of twice background.

Splenocyte Isolation

After $C0_2$ euthanasia of mice, spleens were removed, placed on a 70 μm nylon cell strainer (BD Falcon #352350) in a small petri dish with 3ml RPMI (Mediatech Inc) and crushed with a rubber tipped plunger from a 3 ml syringe. Splenocytes were washed through the cell strainer with 5 ml of RPMI. Cells were recovered and centrifuged at 400 x g for 10 minutes. Supernatant was removed and cells were placed in 5 ml ACK lysing buffer (Quality Biological #118-156-721) for 5 minutes. 10 ml of IgE media (Iscove's DMEM (Mediatech #15-016-CV) + 10% fetal calf serum (Valley Biomedical #BS3032), 1% L.-gutamine (Mediatech #25-005CI), 1% insulin-transferrin-selenium (Mediatech #25-800-CR), and 80ug/ml Gentamicin (Quality Biological #120-098-661)) was added and the cells were centrifuged again at 400 x g for 10 minutes. Supernatant was removed and the cells were resuspended in 10 ml IgE Media. The number of splenocytes was determined by using a Countess automated cell counter system (Invitrogen C10227). Cells were then diluted with IgE media to a concentration of $2X10^6$ cells/ml.

Cytokine Assays

$2X10^6$ splenocytes were cultured in 1 ml aliquots in a 48 well plate at 37°c for 3 days stimulated with either media, 15 μg GutAg, or 15 μg LsAg. After 72 hours, plates were centrifuged at 400 x g for 10 minutes and the supernatant was carefully removed and placed in microtiter tubes. These were centrifuged again at 400 x g for 10 minutes to

remove any residual splenocytes. The supernatant was saved and stored at -20°C. Levels of IL-4, IL-10 and IFN-gamma were obtained with the eBioscience, READY SET GO ELISAS (Cat #88-7044-88, #88-7104-88, 88-7314-88) according to their included protocols.

Proliferation Assay

200,000 splenocytes were cultured in a 100 µl aliquots in a 96 well plate at 37°C for 72 hours. Splenocytes were stimulated with either media, 15 µg/ml GutAg or 15 µg/ml LsAg. Cell proliferation was determined using a BRDU Chemiluminescence assay (Roche #11 669 915 001) according to the manufacturer's protocol. BRDU was diluted 1:100 with IgE media to make the BRDU labeling solution. 10 µl of BRDU labeling solution was added 72 hours after stimulation of splenocytes, and the splenocytes were incubated at 37°C for 17 more hours. Cells were centrifuged at 300 x g for 10 minutes, and the supernatant was removed. A hair dryer was used to completely dry the plates containing splenocytes. 200 µl of FixDenat solution was added to each well for 30 minutes at room temperature. 100 µl anti-BRDU peroxidase (POD) working solution was then added to each well, and plates were incubated for 90 minutes at room temperature. Wells were then washed three times with 200 µl of the included washing buffer with a soaking time of 1 minute each. Finally, 100 µl of substrate solution was added and plates were shaken for 3 minutes. Luminescence was read with a Victor^3 microplate reader (Perkin Elmer). Proliferation indexes were obtained by dividing the luminescence of antigen stimulated wells by those of the media stimulated wells.

Basophil Stimulation

8 week infected BALB/c mice were euthanized with CO_2. Blood was obtained by cardiac puncture with a 22 gauge needle, placed in heparanized tubes (Sarstedt

#41.1503.105), and centrifuged at 400 x g for 10 minutes. Plasma was removed and cells were washed with RPMI, centrifuged again, and resuspended in a volume of RPMI that corresponded with the amount of plasma removed. Cells were then divided into 100 µl aliquots and stimulated with addition of 100 µl RPMI alone as a control or with 100 µl of 4 µg/ml of GutAg or LsAg in RPMI to obtain final concentrations of 2 µg/ml. Samples were incubated at 37°C in 5% CO_2. GolgiStop (BD) was added at a final concentration of 0.7µl/ml after 1 hour and cells were then incubated for an additional 2 hours. Cells were then washed with PBS and centrifuged at 500 x g for 8 minutes. Red blood cells were lysed and the remaining cells fixed with a whole blood lysing reagent kit (Beckman Coulter). Cells were washed twice with 2 ml PBS and blocked overnight at 4°c with 1%BSA/PBS. Cells were stained with anti-IgE FITC (BD Clone R35-72), anti-CD4 PerCP (BD clone RM4-5)), anti-CD45b/B220 PerCP (BD clone RA3-6B2), and incubated at 4°C for 30 minutes. Cells were then permeabilized with 2 ml permeabilization/wash buffer (BD) for 15 minutes at room temperature in the dark. Cells were centrifuged as before, resuspended in 100 µl BD Perm/wash buffer, and stained with anti-IL-4 APC (BD Clone 11B11) for 30 minutes at 4°C in the dark. Cells were washed twice more with PBS and resuspended in 200 µl PBS and analyzed on an LSR II flow cytometer (BD). Analysis was conducted using BD FacsDiva software. Basophils were identified as IgE high and CD4/B220 low cells. All flow cytometry antibodies were titrated prior to use. Cut-offs for determination of activated basophils on the basis of IL-4 positivity were determined using a fluorescence minus one control.

Vaccination

GutAg was mixed with CpG (30 BP Thioate), Alum (Thermoscientific), and PBS to create a mixture containing 10 µg of GutAg, 25 ug CpG, 25 µg alum (.64µl) per 100 µl

of fluid. This mixture was shaken at room temperature for 30 minutes. 100 µl (10 µg of antigen) was injected IP into female BALB/c mice for a total of 3 times at 3 week intervals. Control mice received similar injections of PBS with the same concentration of CpG and Alum, but without antigen. 200 µl of blood was drawn retro-orbitally 2 weeks after each injection for determination of parasite specific antibody levels. Mice were infected with 40 L3s subcutaneously 3 weeks after their last vaccination.

Determination of microfilaria levels and adult worm numbers

8 weeks post infection, mice were euthanized with CO_2. Blood was drawn via cardiac puncture with a 22 gauge needle. 30 µl of blood was then transferred into 1 ml of ACK lysing Buffer (Quality Biological Gaithersburg, MD) and stored at room temperature for 1-3 days for microfilarial counts. This was centrifuged at 400 x g for 10 minutes, and the supernatant aspirated until about 50 µl remained. The pellet was resuspended in the remaining volume and then placed onto a slide and covered with a cover slip. All microfilariae on the slide were counted.

After cardiac puncture, the peritoneal cavity was opened and all aspects of it visually inspected for adult worms with use of a dissecting probe. A small incision was then made in the ventral aspect of the diaphragm and a large tip transfer pipette was used to lavage the pleural cavity with 10 ml warmed RPMI (Mediatech Inc.). The fluid and worms obtained from the lavage were transferred to a petri dish. After lavage, the diaphragm was cut away and inspected for adult worms along with the rest of the pleural cavity. All remaining adult worms that were found were transferred to a Petri dish using a dissecting probe. Worms were separated using fine tipped forceps, sexed, and counted with the aid of a dissecting microscope.

Animal Use

Mice were housed at the Uniformed Services University of the Health Sciences (USUHS) laboratory animal facility and had access to food and water *ad libitum*. All research was conducted under protocols approved by the USUHS Institutional Animal Care and Use Committee.

Statistical Analysis

Immune responses to GutAg and LsAg in the same mice were compared using the non-parametric Wilcoxon Matched Pairs test. The non-paired, non-parametric Mann-Whitney test was used for immunological and worm burden comparisons between vaccinated and unvaccinated mice. All analyses were conducted using GraphPad Prism software (GraphPad, LaJolla).

RESULTS

Microdissection enables purification of *L. sigmodontis* intestinal tracts

To determine whether dissection of adult female *L. sigmodontis* worms results in pure preparations of filarial intestines, components of worms obtained during dissection were stained with hematoxylin and eosin and evaluated histologically. As seen in figure 1, microdissection enables purification of intestinal tracts from adult female *L. sigmodontis* worms.

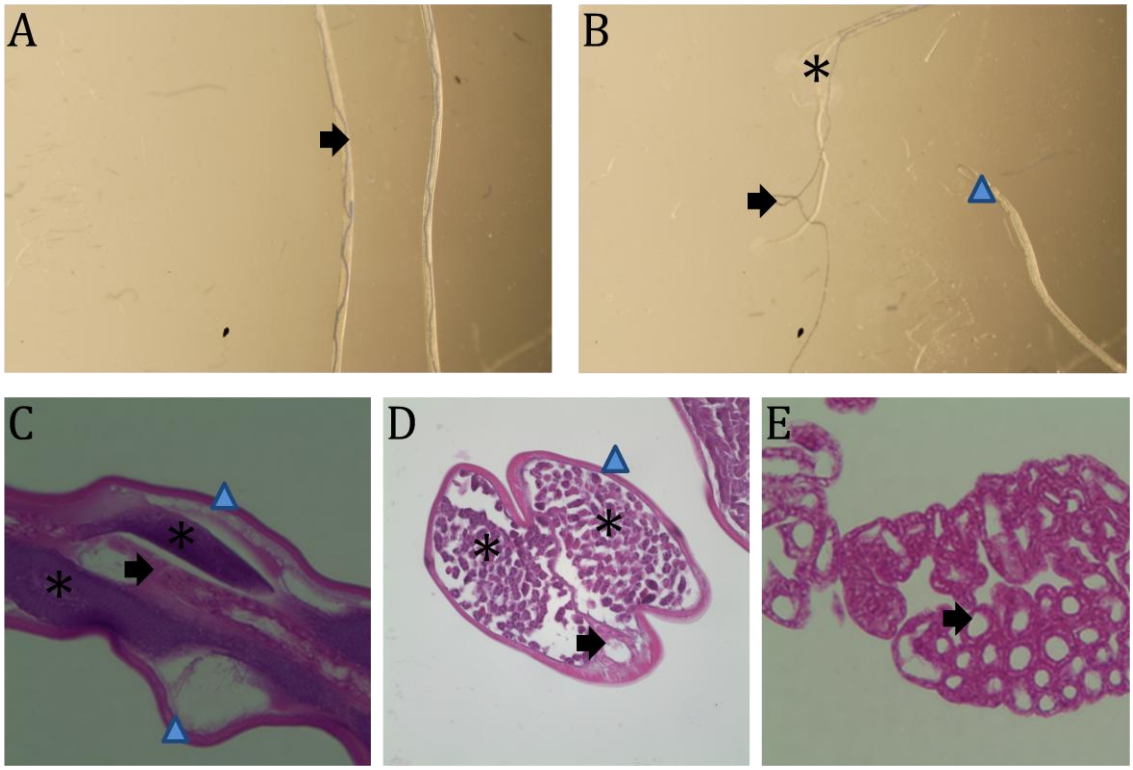


Figure 1: Gross anatomy and histology of adult female *Litomosoides sigmodontis*. Gross anatomy as seen from the dissecting scope prior to (A) and after (B) mechanical rupture of the cuticle (25X). Longitudinal (C) and Cross sectional (D) views of adult female worm prior to dissection (H&E,400X). (E) Purified intestinal tract from adult female worm with characteristic single layer of cuboidal cells. The intestinal tract is viewed after purification repeatedly folded back on itself (H&E, 400X). (Black arrow=Gut, Asterisk=Uterine Tube, Blue triangle=Cuticle)

Infected mice display lower immune responses to GutAg than to LsAg

To determine the extent to which intestinal antigens are "hidden" from the immune system during *L. sigmodontis* infection, humoral and cellular responses toward parasite antigens were measured in mice with active infections (8 weeks PI) and previous infections (22 weeks PI). IgG antibody titers to either the soluble fraction of the entire worm (LsAg), or the soluble fraction of the worm digestive tract (GutAg) were determined by ELISA. Originally, we hypothesized that currently infected mice would display relatively weak adaptive immune responses to GutAg compared to LsAg because of sequestration of these antigens from the host immune system. We also hypothesized that 22 weeks PI, after worm death and degradation had occurred, that these titers would increase as the immune system may have had an opportunity to interact with antigens from the worm digestive tracts. However, we found that GutAg specific immune responses were low both during and after infection (Figure 2).

As seen in figure 2A, mice developed high titers of LsAg specific IgG antibodies during active infection which dropped after worm clearance had occurred. At both time points, these titers were significantly greater than GutAg specific IgG titers. In contrast to IgG, LsAg and GutAg IgE titers were similar during active infection. In previously infected mice, however, GutAg-specific IgE titers were significantly lower than those against LsAg (Figure 2B).

Cellular responses were also generally lower against GutAg than LsAg. At both time points evaluated, splenocyte proliferation and production of IL-4 and IFN-γ were lower in response to GutAg than to LsAg (Figure 3A-C). No differences were observed with respect to splenocyte IL-10 production (Figure 3D).

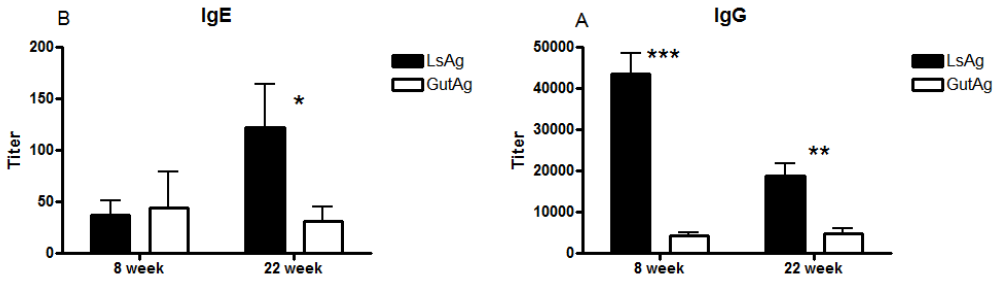


Figure 2: Antibody titers to soluble antigens preparations from entire L. sigmodontis worms (LsAg) or their digestive tracts (GutAg) in currently infected (8 week) or previously infected (22 week) BALB/c mice. Shown are composite results from two independent experiments, each of which had 5 animals per group. (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$)

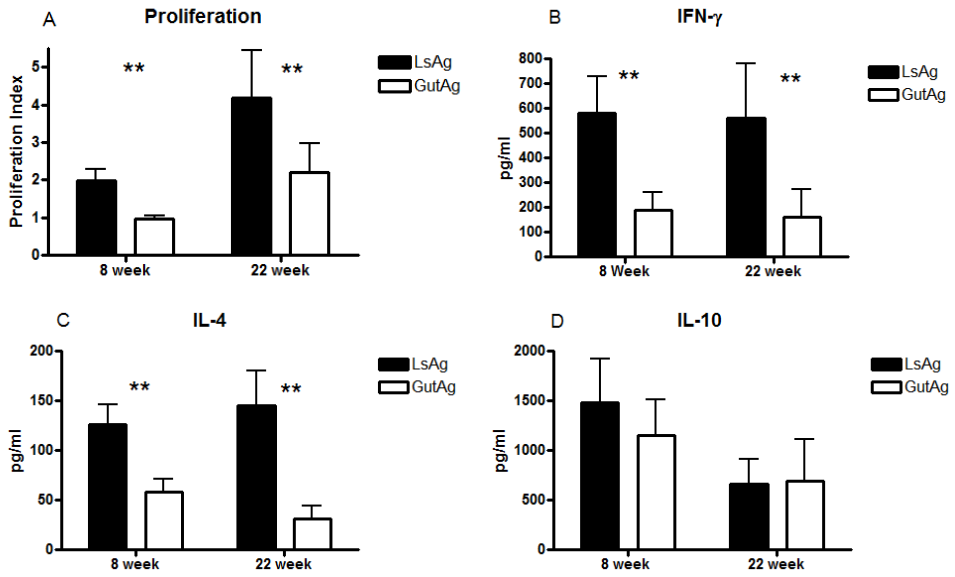


Figure 3: Splenocyte responses in 8 week and 22 week infected BALB/c mice when cultured for 3 days at 2 million cells/ml and stimulated with 15 µg/ml LsAg or GutAg. (A) Splenocyte proliferation as measured by incorporation of BRDU. (B-D) Cytokine levels in the supernatant of splenocytes as measured by ELISA. Shown are composite results from two independent experiments, each of which had 5 animals per group. (*=$p<0.05$, **=$p<0.01$)

GutAg induces basophil activation in *L. sigmodontis*-infected mice

To assess the allergic potential of GutAg, we assessed the extent to which GutAg activates basophils during infection. As basophils are allergy effector cells that are activated by cross-linking of surface-bound IgE antibodies, basophil activation tests are immunological markers for allergic responses (59). To measure basophil activation, peripheral blood cells were obtained from 8 week infected mice, stimulated with either GutAg or LsAg, and then evaluated for the production of IL-4 by flow cytometry. As seen in figure 4, GutAg stimulation induced equivalent activation of basophils as LsAg (% IL-4+ basophils =33% after media stimulation vs 60% after both GutAg and LsAg stimulation). This result, along with the development of GutAg-specific IgE antibodies, suggests that vaccination with the soluble fraction of filarial intestinal antigens may not circumvent the issue of allergic responses in exposed individuals.

Vaccination Results in Increased anti-Gut IgG Production, and Balanced IgG1/IgG2A Responses

Since antibody responses to GutAg remained fairly low throughout infection, we tested whether vaccination with GutAg could induce robust anti-GutAg antibody production. Because prior filaria vaccine studies have shown that both type 1 and type 2 responses can have protective effects against filaria infection (2; 58), we chose to use a combination of CpG and Alum as adjuvants to induce a broad-based immune response. As seen in figure 5A, mice vaccinated three times with 10 μg GutAg developed anti-GutAg IgG titers over five fold higher than previously infected mice. Furthermore, vaccinated mice produced a more balanced ratio of IgG subtypes, including both IgG1 and IgG2A. Conversely, previously infected mice produced almost exclusively the

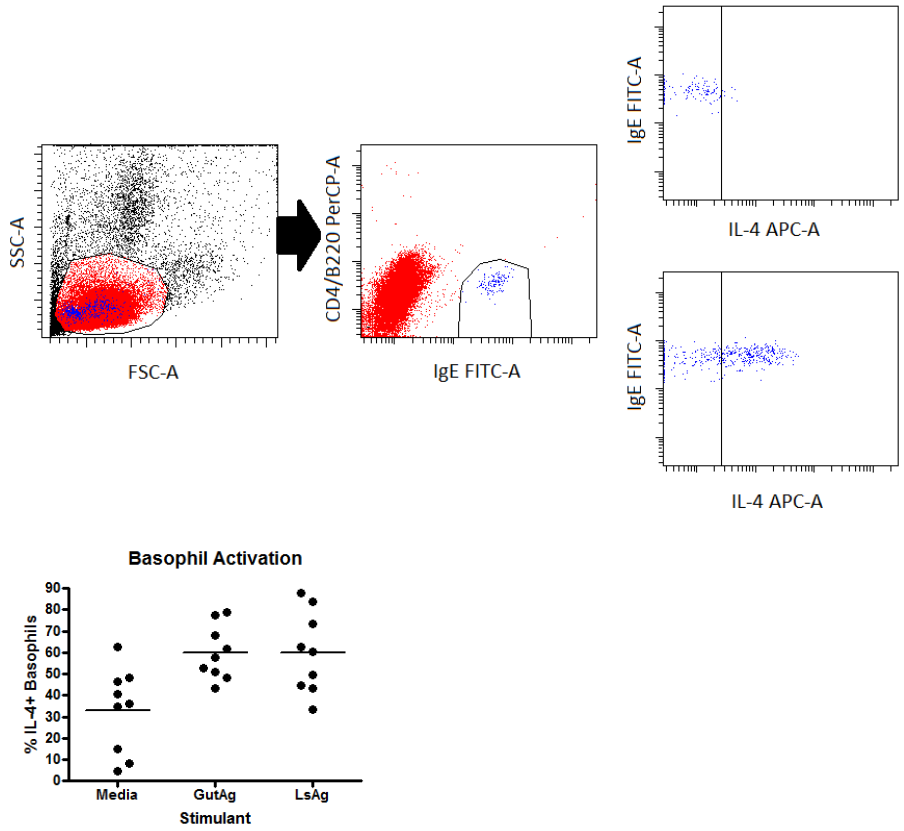


Figure 4: Basophil responses to LsAg or GutAg in 8 week infected mice. (A) Cells were initially gated by forward and side scatter characteristics (left panel). Basophils were identified by high expression of IgE and low expression of CD4 and B220 (Mid Panel). IL-4 expression in unstimulated (right top) and stimulated basophils (right bottom). (B) Percentages of basophils positive for IL-4 after stimulation with 2µg/ml LsAg or GutAg. Shown are composite results from two independent experiments, one which had 4 animals per group and one which had 5 animals per group.

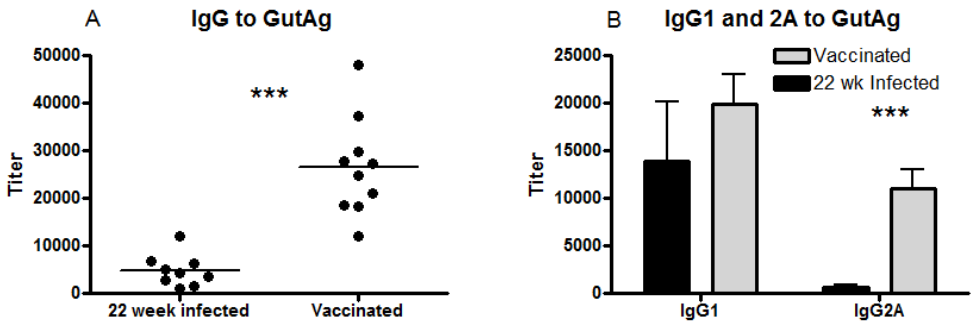


Figure 5: Anti-GutAg antibody titers in 22 week infected mice and mice vaccinated with GutAg+CpG/Alum as measured by ELISA. (A) Total anti-GutAg IgG titers. (B) IgG1 and IgG2A titers. Shown are composite results from two independent experiments, each of which had 4-5 animals per group. (***=$p<0.001$)

TH_2-associated IgG1 subtype which provides very little complement fixation (74; 105) (Figure 5B).

Vaccination with Gut Antigen Fails to Protect Against Challenge Infection.

In order to determine the efficacy of a vaccine comprised of GutAg, mice were vaccinated three times with either GutAg or saline with CpG and Alum. Three weeks after the last vaccination, mice were infected with 40 infectious larvae. Eight weeks PI, adult and microfilaria parasite burdens were determined. No protection was garnered from vaccination with GutAg, as vaccinated mice harbored 19.4 worms on average, whereas non-vaccinated mice only harbored an average of 18.3 worms (Figure 6A). Microfilaria burdens in vaccinated animals were also similar to those of non-vaccinated animals (Figure 6B).

DISCUSSION

Filarial worms cause diverse and debilitating diseases throughout much of the tropics, and a vaccine would aid in both control and possible elimination of these diseases. Vaccine development in filariasis is complicated by a lack of protective immunity during infection and by possible allergic responses to worm antigens after exposure (42-44; 77; 78). Because the digestive tract is sequestered within the worm, and thus may be immunologically "hidden" during the course of normal infection, vaccination against intestinal antigens has been proposed as a possible means of overcoming these obstacles(49; 65; 99).

Studies using the nematode models of *Haemonchus* and *Dirofilaria* have shown that infection elicits weak antibody responses to parasite intestinal antigens and that vaccination with intestinal antigens induces high antibody responses and protection

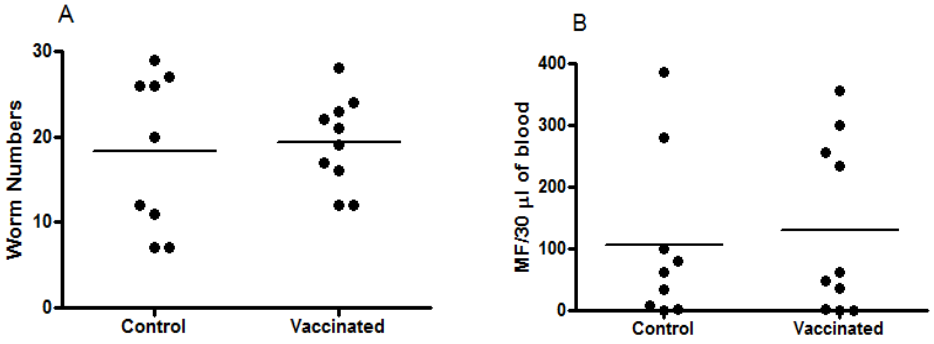


Figure 6: Adult worm (A) and microfilaria (B) parasite burden of vaccinated and control mice 8 weeks after infection with *L. sigmodontis*. Vaccinated mice were given 3 IP injections of GutAg+CpG/Alum prior to infection; control mice were given injections of PBS with Cpg /Alum. Shown are composite results from two independent experiments, each of which had 4-5 animals per group.

against infection (99; 132). However, neither of the models used to date have been ideal vaccine models for human filariasis. *Haemonchus* is a non-filarial nematode parasite of the intestinal tract, and the *Dirofilaria* experiments utilized a non-permissive host to determine vaccine efficacy. Thus, in this study we used the permissive *Litomosoides sigmodontis* BALB/c model of filariasis to investigate the vaccine potential of filarial intestinal antigens.

Our studies using the soluble fraction of filaria intestinal antigens demonstrate that natural infection results in allergic sensitization against filaria intestinal antigens and that vaccination with these antigens fails to protect against challenge infection despite the development of high antibody titers.

Our first set of experiments evaluated the immunological responses to GutAg in actively infected and previously exposed mice to determine the extent to which these antigens are immunologically 'hidden' during infection. Similar to other models, the IgG responses that developed to GutAg were relatively low. Furthermore, while splenocytes of infected mice responded to GutAg, cytokine production and proliferation of splenocytes were generally lower to these antigens than to LsAg. Overall, the lower humoral and cytokine responses to GutAg when compared to LsAg suggest that a large portion of the antigens contained within the digestive tract of the worm may remain hidden from the immune system throughout infection. It is important to note, though, that the lower antibody and cellular immune responses observed towards GutAg may have also been due to a quantitative phenomenon whereby the more antigenically diverse LsAg induced greater immune responses than GutAg because of a higher number of distinct antigens present in the mixture.

In terms of allergic sensitization, our study clearly shows that GutAg is not immunologically hidden. Both actively infected and previously infected mice produce GutAg-specific IgE. This IgE is functional, as basophil activation assays demonstrated that GutAg was as effective as LsAg in activating basophils of infected mice. These results suggest that vaccination with the soluble fraction of filarial intestinal antigens would have the potential to induce allergic responses in previously infected individuals.

Vaccination of mice with GutAg adsorbed to CpG and Alum resulted in high titers of GutAg-specific IgG1 and IgG2a antibodies. Despite this, GutAg vaccination failed to protect against infection. This either occurred because the titer of antibodies induced was not high enough to enable protection, or because GutAg specific antibodies simply do not have the capability to protect against infection in this model. One can speculate that GutAg specific antibodies are unable to interact with their specific antigens in this model because of either a lack of antibody ingestion by the parasite or because antibodies are degraded by proteases within the worm digestive tract. Despite the negative results of this study, however, we suspect that some intestinal tract antigens do have the potential to function as effective filaria vaccine candidates. First, there is direct evidence that some antigens present in the intestinal tracts of filariae can serve as effective vaccine candidates. Preliminary evidence from a proteomic study we are conducting demonstrates that glutathione-S-transferase and superoxide dismutase are present within the digestive tract of filarial worms. As both of these antigens induce protection when used to vaccinate jirds against filariasis (36; 122), it is clear that some antigens located in filarial intestinal tracts can function as effective vaccine antigens. Second, it is unlikely that the lack of protection in this model is due to a lack of worm

antibody ingestion as *L. sigmodontis* worms ingest blood between 28-56 days PI (9), providing some opportunity for antibody ingestion. It is also likely that *L. sigmodontis* ingests antibodies directly from the pleural fluid. Thus, the most likely reason for the lack of protection observed in this study is that no protective antigen within the intestinal fraction was given in a high enough dose to elicit a protective immune response. This is similar to what has been shown in Jirds vaccinated against *Brugia* species. Vaccination with the soluble fraction of adult *Brugia pahangi* does not elicit any significant decrease in adult worm burden, while vaccination with *Brugia malayi* adult somatic antigen has shown to provide only 25% protection against challenge infection (75; 150). Despite this, vaccination with specific antigens contained in the adult worms such as myosin can give much higher levels of protection (150). There are no published reports on the use of LsAg as a vaccine in mice, but albino rats are not protected against infective larvae or adult stages after vaccination with LsAg (100). Furthermore, preliminary research in our lab suggests that this vaccine approach is not protective in mice (personal observation).

In conclusion, in this study we demonstrated that the soluble fraction of filarial intestinal antigens elicits allergic sensitization during infection and fails to protect when used as a vaccine adsorbed to alum and CpG. While disappointing in terms of filaria vaccine development, these findings, do not prove that there are no antigens within filarial intestinal tracts that could be both protective and safe. One of the major limitations of this study was obtaining enough GutAg for all of the studies involved. Indeed, it took a single investigator (C.P.M.) more than 350 hours to dissect the 3000+ worms used to obtain enough purified filarial intestinal tracts for the experiments done in this study. As such, increasing the concentrations of GutAg used for vaccination or

testing various fractions of GutAg are likely not feasible approaches for further investigating the potential of filarial intestinal antigens as vaccine candidates. Consequently, we are currently conducting a proteomic analysis to identify antigens present only within the intestines of filarial worms. Recombinant expression of such antigens or use of DNA vaccines can then be tried in future studies to explore the potential of specific intestinal antigens to function as effective vaccine candidates.

Chapter 3 Vaccination with mammalian derived irradiated larvae results in a protective immune response that is not antibody dependent

INTRODUCTION

Filarial worms are responsible for highly morbid diseases such as elephantiasis and African River Blindness in humans. These diseases, while not typically fatal, are detrimental to the ability of individuals to work and live a normal life. Development of safe and effective vaccines against filariasis would help ongoing efforts to control and eliminate these diseases (11).

To date, the most effective vaccine approach against filariasis in animal models has been use of irradiated L3 stage larvae. Vaccination with irradiated larvae has been shown to be an effective approach in 16 different animal models of filariasis, and has induced levels of protection as high as 98% in some models (137). Using the *Litomosoides*/BALB/c model, it has been shown that both antibodies and IL-5 are important factors in an effective immune response, and that protection occurs very early after challenge infection (83; 96; 97). While effective in a laboratory setting, the complicated life cycles of filarial parasites and our inability to maintain the life cycle in *in vitro* culture make use of irradiated L3 vaccines in people not feasible.

The goals of this study were to determine whether soluble adult filarial antigens could function as effective vaccine candidates and to further elucidate the mechanism by which irradiated L3 vaccination protects. Soluble adult antigens have shown mild protection in animal models using *B. malayi,* and provided protection against microfilaremia in albino rats infected with *L. sigmodontis* (100; 150). In this study, we initially vaccinated mice with the soluble fraction of adult worm antigen using CpG/Alum as an adjuvant to induce both type 1 and type 2 responses. Although this vaccine approach was not protective, it allowed us the opportunity to compare the

immune responses of mice given an effective filarial vaccine (irradiated larvae) with those given the ineffective LsAg vaccine. Our studies demonstrate that 1) skewing the immune response induced by LsAg strongly towards a Th2 phenotype, as occurs with irradiated larval vaccination, does not enhance protection of LsAg vaccination, 2) clearance of worms in our irradiated L3 vaccination model occurs predominantly after the worms have reached the pleural space, and 3) irradiated larval vaccination does not require antibodies to be effective.

MATERIALS AND METHODS

Animal Use

BALB/c mice (NCI) and antibody deficient J_H Mice (BALB background Taconic mouse model 001147) were used to carry out research. All research was conducted in compliance with protocols approved by the Uniformed Services University of the Health Sciences (USUHS) institutional Animal Care and Use Committee. All animals were housed at the USUHS laboratory animal facility and had constant access to food and water.

Infection

Infectious larvae were obtained from jirds 4 days post infection. Jirds were exposed to mites harboring infectious larvae. 4 days later, after infectious larvae had migrated to the pleural cavity, these jirds were euthanized by CO_2 asphyxiation and dissected to open the peritoneal cavity. After the internal organs were moved in order to visualize the diaphragm, a 0.5 cm incision was made in the diaphragm, and the pleural cavity was lavaged with 10 ml of warm (37°C) RPMI (Mediatech inc. Manassas, VA). With the aid of a dissecting scope, L3s were counted, aspirated with a P20 micropipettor, and placed into a syringe. Mice were then infected by subcutaneous injection of 40 L3s into the nape of the neck using a 22 gauge needle.

Antigen Preparation

Adult *L. sigmodontis* were obtained from chronically infected jirds. In brief, Jirds were dissected to reveal the peritoneal cavity, which was visually inspected for parasites. Afterward, the diaphragm was cut away using scissors, and the pleural cavity and pericardial sac were examined for parasites. Adults were manually extracted with a surgical probe, placed in 37°C RPMI, examined under the dissecting microscope and separated from any host tissues. After being rinsed with RPMI, adults were stored at -20°C until use. When making LsAg, adult *L. sigmodontis* were thawed and placed into "D" tubes from Biopulverizer System 1 (MP Biomedicals Cat 6750-200), and were homogenized by running on a FastPrep-24 System (MP Biomedicals) at 4.0 M/s^2 for 20 seconds for a total of three runs on the machine. Between runs, the homogenate was centrifuged at 16,100 x g for 1 minute, the supernatant was aspirated off and saved, and 1 ml of PBS was added to the pellet (still in the "D" tube). The saved supernatants were centrifuged at 15,000 x g to remove any residual non-soluble material. The supernatants were aspirated with a pipette, saved, and then filtered through a 0.22 micron syringe filter. This sterilized soluble fraction of the adult worms was stored at -20°C until use in experiments.

Vaccination

Vaccine strategies included vaccination with LsAg, or vaccination with irradiated larvae (iL3). LsAg vaccines were performed in three different ways: 1) Vaccination with 10 µg LsAg using 25 µg CpG (30BP Thioate), and 25 µg Imject Alum (Thermoscientific) as an adjuvant, 2) vaccination with 10 µg LsAg using 2mg Alum as an adjuvant, or 3) vaccination with 50 µg of LsAg using 2 mg Alum as an adjuvant. Mice were vaccinated with LsAg 3 times by intraperitoneal (IP) injection at 3 week intervals for CpG/Alum and

1 week intervals for Alum only. iL3 vaccinated mice were given 3 weekly subcutaneous injections of 25 irradiated L3 larvae (iL3, 45 krad using cobalt 60 irradiator).

Pre-incubation in immune sera

L3s were obtained from jirds as above. L3s were separated into aliquots of 200 worms/400 µl RPMI. These aliquots were mixed with 400µl of either RPMI or mouse plasma (obtained from iL3 vaccinated mice, or previously infected mice). These were mixed thoroughly, incubated for 2 hours at 37°C, and then injected subcutaneously into mice as above.

Splenocyte suspensions

Mice were euthanized with CO_2 asphyxiation, and their spleens were removed and placed in 3ml RPMI. Spleens were placed on a 70 µm Nylon Cell strainer (BD 352350) and crushed with a 3 ml syringe plunger (BD 309656). The cell strainer was washed with 10 ml RPMI. This cell suspension was centrifuged at 400 x g for 10 minutes, and cells were resuspended in 1 ml ACK lysing buffer (Quality Biological 118-156-721) for 5 minutes to lyse red blood cells. The cells were then washed with 9 ml IgE media (Iscove's DMEM (Mediatech #15-016-CV) + 10% fetal calf serum (Valley Biomedical #BS3032), 1% L-glutamine (Mediatech #25-005CI), 1% insulin-transferrin-selenium (Mediatech #25-800-CR), and 80ug/ml Gentamicin (Quality Biological #120-098-661)). Cells were then centrifuged at 400 x g for 10 minutes and resuspended in IgE Media. Splenocyte concentration was determined with a Countess automated cell counter system (Invitrogen C10227).

Measurement of Splenocyte Proliferation Using BRDU

100 µl of splenocytes were cultured in triplicate at $2x10^6$ cells/ml and stimulated with media alone, 15 µg LsAg, or 2.5 µg anti CD3 and 2 µg anti CD28 for 72 hours at 37°C. Cell Proliferation was determined using BRDU chemiluminescence assay (Roche

#11 669 915 001). 10 µl of BRDU was added after 72 hours (diluted 1:100 in IgE media) and allowed to incubate with splenocytes for 17 hours. Splenocytes were then centrifuged at 300 x g for 10 minutes, decanted, and dried with a hair dryer. 200 µl of FixDenat solution was added for 30 minutes and then poured off. 100 µl anti-BRDU peroxidase (POD) working solution was added, and plates were incubated for 90 minutes at room temperature. Each well was washed 3 x with 200 µl washing buffer and allowed to soak for 1 minute. 100 µl of substrate solution was added and plates were shaken for 3 minutes. Plates were read with a Victor^3 microplate reader (Perkin Elmer). Proliferation index was determined by dividing the luminescence of stimulated wells by those of media stimulated wells.

Measurement of Cytokines with ELISA.

Splenocytes were cultured in a 48 well culture plate in 1 ml aliquots of 2 million cells. These were stimulated for 72 hours at 37°C with media, 15 µg/ml LsAg, or 2.5µg/ml anti CD3 and 2µg/ml anti CD28. After 72 hours, plates were centrifuged at 400 x g for 10 minutes, and the supernatant was aspirated off and stored at -20°C. The supernatant was tested for IL-4, IL-5, IFNγ, and IL-10 using eBioscience Ready Set Go ELISAS (Cat #88-7044-88, #88-7104-88, #88-7314-88, #88-7054-88) according to their included protocols, except that half-well ELISA plates were used, and all volumes were cut in half.

Determination of Parasite Burden

Mice were euthanized with CO_2, and then were dissected to reveal the peritoneal cavity, which was visually inspected for parasites. The liver was moved aside to display the diaphragm, which was cut on the ventral aspect. The pleural cavity was then lavaged with 10 ml pre-warmed (37°C RPMI). After lavage, the diaphragm was cut away with

scissors, and the pleural cavity was inspected for parasites. After adults were recovered manually and by lavage, they were separated and counted with the aid of a dissecting microscope.

Determination of Antibody Titers

2 weeks after the last vaccination, blood was obtained from mice, placed in a plasma separator tube (BD CAT#365958), and centrifuged at 16,100 x g for 1 minute. Plasma was aspirated off and stored at -20°C until use. Parasite specific antibody titers were measured in 96 well half area EIA/RIA plates (Corning#3690). Plates were coated overnight at 4°C with 50 µl of 2 µg/ml LsAg diluted in PBS. Plates were washed with washing buffer (PBS with 0.025% Tween 20 (Acros Organics) using an ELX 405 microplate washer (Biotek instruments, Winooski VT), and then blocked for 2 hours at room temperature with blocking buffer (PBS with 5% BSA and .05% tween 20). For IgG, IgG1, and IgG2A titers, plasma was diluted with assay diluent (PBS, 1% BSA, 0.05% tween 20) 1:25 for the top dilution, followed by 7 5-fold serial dilutions. For IgE titers, plasma eluted of IgG was diluted 1:4 for top dilution followed by serial 2 fold dilutions. After the plate was washed again, 50 µl of serially diluted plasma was added to wells and incubated for 2 hours at room temperature. 50 µl of detection antibody (anti mouse IgG (diluted 1:1000), IgE (2 µg/ml) IgG1 (2 µg/ml), or IgG2A (2 µg/ml) see antibody section for more information) was added and incubated at room temperature for 1 hour. For the IgE, IgG1 and IgG2A ELISAs, plates were washed and then incubated with 50µl streptavidin conjugated alkaline phosphatase (Jackson Immunoresearch #016-050-084) diluted 1:1000 in assay diluent. After the final wash, 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich N9389) was then added at a concentration of 1mg/ml in sodium carbonate buffer (KD Medical Cat CUS-0242) and incubated at room

temperature for 35 minutes. A Victor^3 microplate reader (Perkin Elmer) was used to read OD values at 405nm. These were then graphed with Graphpad and a four parameter curve was measured for each plasma sample. This four parameter curve was used to determine the dilution of plasma that would provide an OD of 2x background which was used as the titer.

CD-4 Depletion:

500µg GK1.5 (BioXcell BE0003-1) antibody was injected IP into vaccinated mice 2 days prior to challenge infection, and weekly thereafter until sacrifice. Flow cytometry was performed on blood (both 1 day prior to infection, and at time of euthanasia) and pleural cells (at time of euthanasia) to verify depletion of $CD4^+$ cells.

Antibodies Used:

Depletion: Rat Anti Mouse CD-4 Clone GK1.5 BioXcell Cat BE0003-1

ELISA: Biotin Rat anti-mouse IgG1 BD Cat 550331, Biotin Rat anti mouse IgG2A BD Cat 550332, Biotin Rat anti mouse IgE Clone R35-118 BD Cat 553419, ALK-Phos Goat anti mouse IgG (Mabtech CAT3310-04).

Stimulation: Anti Mouse CD3e Clone 17A2 EBioscience Cat 14-0032-86, Anti Mouse CD28 Clone 37.51 Ebioscience Cat 16-0281-85

Flow Cytometry: Rat anti-mouse CD4 PERCP Clone RM4-5 BD 553052

Statistics

Mann-Whitney tests were used to determine vaccine efficacy and significance of differences in immune responses in iL3 vaccinated compared to LsAg vaccinated mice.

Results

Mice vaccinated with LsAg adsorbed to CpG/Alum are not protected against future infection, whereas mice vaccinated with irradiated larvae undergo a major decline in worm burden 1-4 weeks after infection.

As both Th1 and Th2 inducing adjuvants have shown efficacy in prior animal studies of filaria vaccines, we initially tested whether LsAg vaccination is protective in

the *Litomosoides sigmodontis* model using a combination adjuvant of CPG with alum. The combination we utilized for the adjuvant has been shown to induce mixed immune responses (39). For the study, BALB/c mice were vaccinated with 10 μg LsAg three times using the CpG ODN and a low concentration of Alum as adjuvant. As seen in Figure 7A, mice vaccinated with this regimen obtained no significant protection against *L. sigmodontis* 8 weeks after infection. This is in stark contrast to mice vaccinated with irradiated larvae, which demonstrated an 80% reduction in worm burdens at 8 weeks PI (Figure 7B). In contrast to other investigations with irradiated L3 vaccination, which have found rapid reductions in worm burden just days after challenge infection, in this study we observed that the majority of worm clearance observed in mice vaccinated with irradiated L3s occurs between 1 and 4 weeks post-challenge (Fig. 7C).

Splenocytes from mice vaccinated with irradiated larvae exhibit greater proliferation and release of type 2 cytokines in response to LsAg than mice vaccinated with LsAg plus CpG/alum.

In order to determine key differences in cellular immunity between an effective and an ineffective immune response, we measured cytokine production and proliferation of splenocytes from PBS plus CPG/alum vaccinated, LsAg plus CPG/alum vaccinated, and iL3 vaccinated mice in response to LsAg. Mice vaccinated with irradiated larvae exhibited a proliferation index 2.8 times higher than that exhibited by mice vaccinated with LsAg (6.69 average compared to 2.41 average, figure 8A). Similarly, levels of both

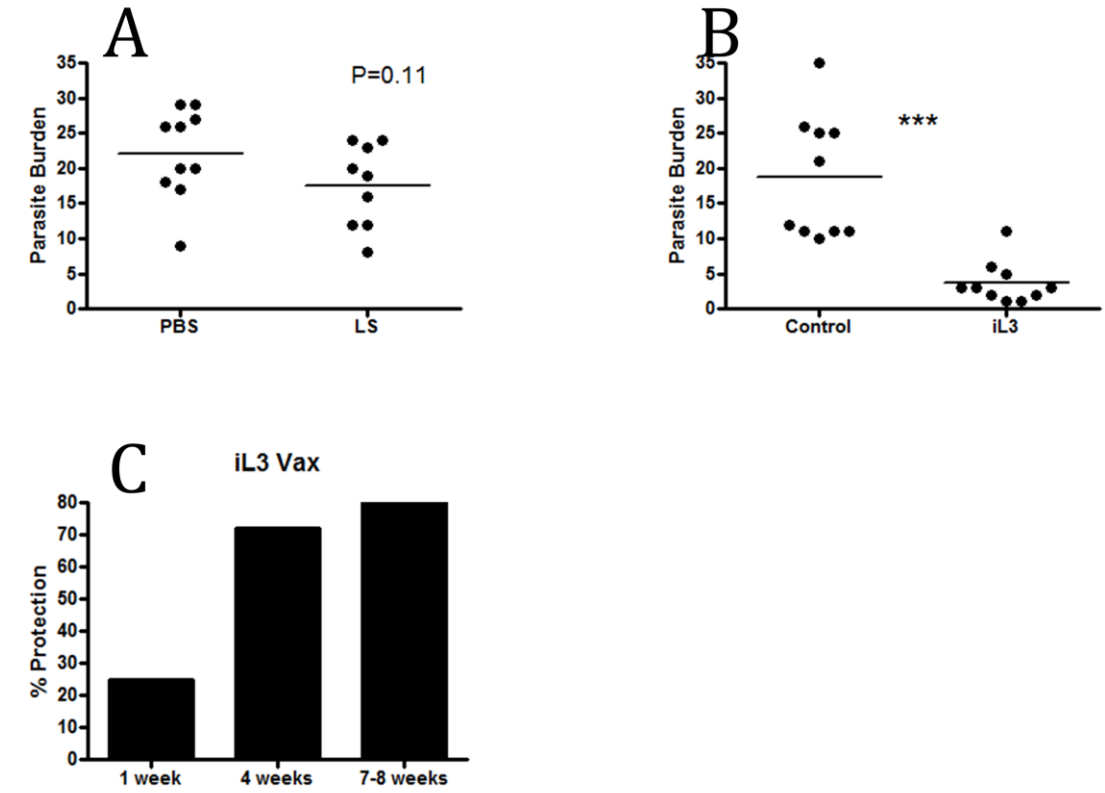


Figure 7: Protection against L. sigmodontis in BALB/c mice vaccinated with LsAg or irradiated L3s. A) Parasite burdens 8 weeks after challenge infection with 40 L3s in mice vaccinated 3 times with either PBS plus CPG/alum (PBS) or 10 μg of LsAg plus CPG/alum (LS). B) Parasite burdens 7-8 weeks after challenge infection with 40 L3s in control unvaccinated mice (control) or in mice vaccinated 3 times with 25 irradiated larvae (iL3s). C) Protection in mice vaccinated with irradiated larvae compared to control mice at 1 week, 4 weeks and 7-8 weeks post infection. Each protection experiment is a composite of two individually performed experiments. Some data in (B and C) is derived from historical data in our laboratory. *** $p < 0.001$

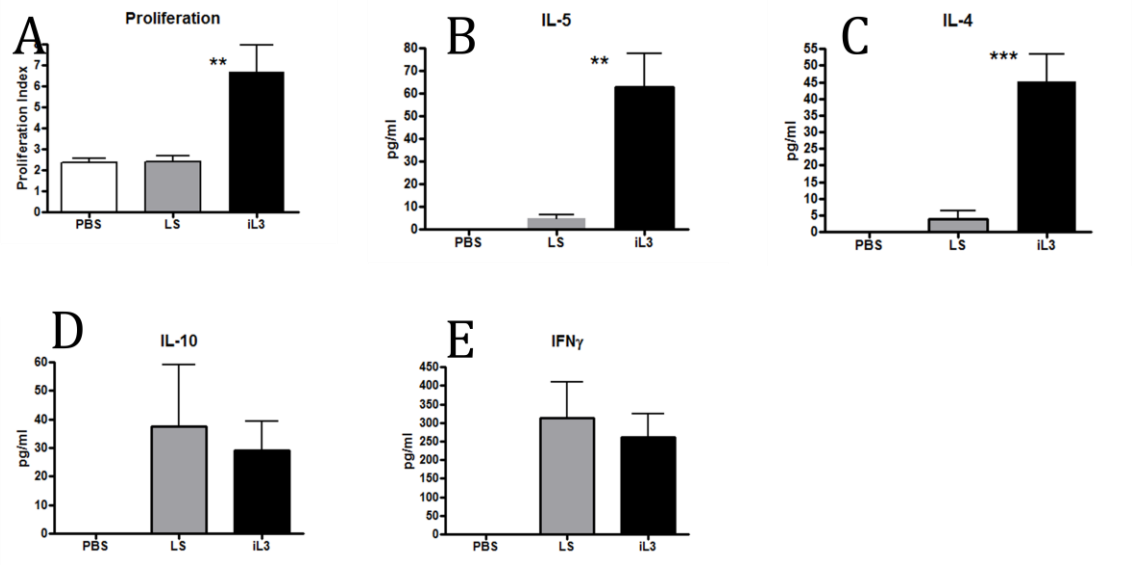


Figure 8: Immunologic responses of splenocytes from BALB/c mice vaccinated with PBS plus CPG/alum (PBS), LsAg plus CPG/alum (LS), or irradiated larvae (iL3s) after in vitro stimulation for 3 days with 15 µg/ml LsAg. (A) Splenocyte proliferation as measured by incorporation of BRDU. (B-E) Cytokine concentration above media stimulation in culture supernatants. ** $p < 0.01$ *** $p < 0.001$

IL-5 and IL-4 were significantly higher in iL3 vaccinated mice compared to LsAg vaccinated mice. In contrast, IL-10 and IFNγ levels were similar between both vaccinated groups (Figure 8 B-D).

Mice vaccinated with iL3s develop a more type 2 skewed antibody response than mice vaccinated with LsAg plus CpG/alum

Consistent with cellular immune responses, antibody responses in mice vaccinated with iL3s exhibit a greater type 2 skew against parasite antigen than mice vaccinated with LsAg plus CPG/alum. Parasite-specific IgG, IgG1, and IgG2a levels were greater in animals vaccinated with LsAg plus CPG/alum than those vaccinated with iL3s (Figs. 9a-c). While we have previously shown that iL3 vaccination induces antibodies that react to LsAg, the greater IgG levels detected against LsAg in LsAg plus CPG/alum vaccinated mice are not too surprising given those mice were directly vaccinated with LsAg. However, the ratio of parasite-specific IgG1:IgG2a was significantly greater in iL3 vaccinated mice (Fig. 9d), and only mice vaccinated with iL3s developed detectable LsAg-specific IgE (Fig. 9e).

Vaccination with 10 or 50 µg LsAg and high levels of Alum induces a type 2 immune skew, but is not protective against future infection.

As the protective iL3 vaccine regimen predominantly results in type 2 cellular and humoral immune responses, we next evaluated whether LsAg could induce protective immunity if given with an adjuvant that skews towards type 2 immunity. To test this, mice were vaccinated with LsAg in combination with a high dose of Alum alone as adjuvant. In contrast to the CpG/Alum combination, in which only 25 µg of alum was used, in this experiment 2mg of alum was used in each vaccine dose, which is typical of vaccine protocols that use Alum alone as an adjuvant. We also attempted to induce a higher immune response by increasing the amount of LsAg to 50 µg per injection.

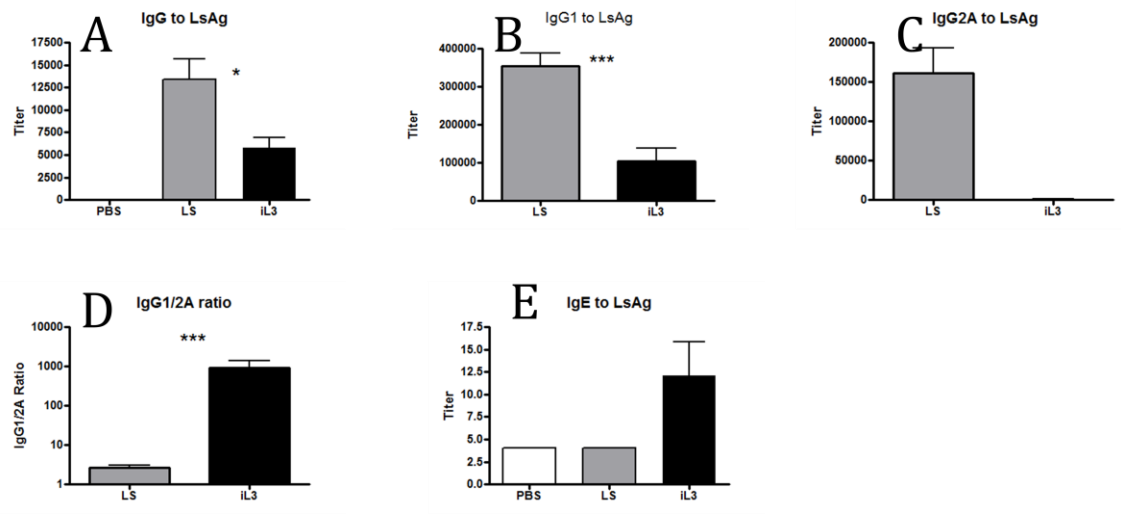


Figure 9: Antibody responses in mice vaccinated with LsAg or irradiated larvae. LsAg-specific total IgG (A), IgG1 (B), and IgG2a titers in plasma of mice vaccinated with PBS plus CPG/alum (PBS), LsAg plus CPG/alum (LS), or irradiated L3s (iL3s). D) Ratio of LsAg-specific IgG1/IgG2A titers. E) LsAg-specific IgE Titers. * $p < 0.05$, *** $p < 0.001$

with the higher amount of alum as an adjuvant induced more of a type 2 skew with levels of IL-5 and IL-4 similar to those seen in iL3 vaccinated mice (Figure 10 B-C), but high amounts of IL-10 and low amounts of IFNγ. Vaccination with this protocol also induced high levels of IgG, with a high IgG1/2A ratio. Despite the Type 2 skew induced by this vaccination protocol, these mice exhibited no protection against challenge, as evidenced by parasite burdens 8 weeks PI (Figure 10H).

Previously infected mice display insignificant levels of protection compared to primarily infected animals.

Since protection is not garnered simply from skewing toward an immune response, it is likely that there are specific antigens that are being targeted after iL3 vaccination compared to LsAg vaccination. These protective antigens may be present in the L3 or L4 stage of the worm, as evidenced by the timeframe of killing after iL3 vaccination. Until the present it has been unclear whether any animal previously exposed to early antigens in the *L. sigmodontis* model would be protected against future infection. In order to determine this, we infected animals with 40 L3s, allowed at least 22 weeks for the infection to clear, and then reinfected these animals. Parasite burdens were compared to age matched controls that received only the challenge infection. Previously infected animals exhibited no protection within the first week after secondary infection. At 4 weeks post challenge, the previously infected mice exhibited 37% less parasites than the control group, but this was not statistically significant (Figure 11A). Splenocyte responses in mice 22 weeks post infection showed moderate levels of proliferation, and production of both type 1 and type 2 cytokines (Fig 11B, C).

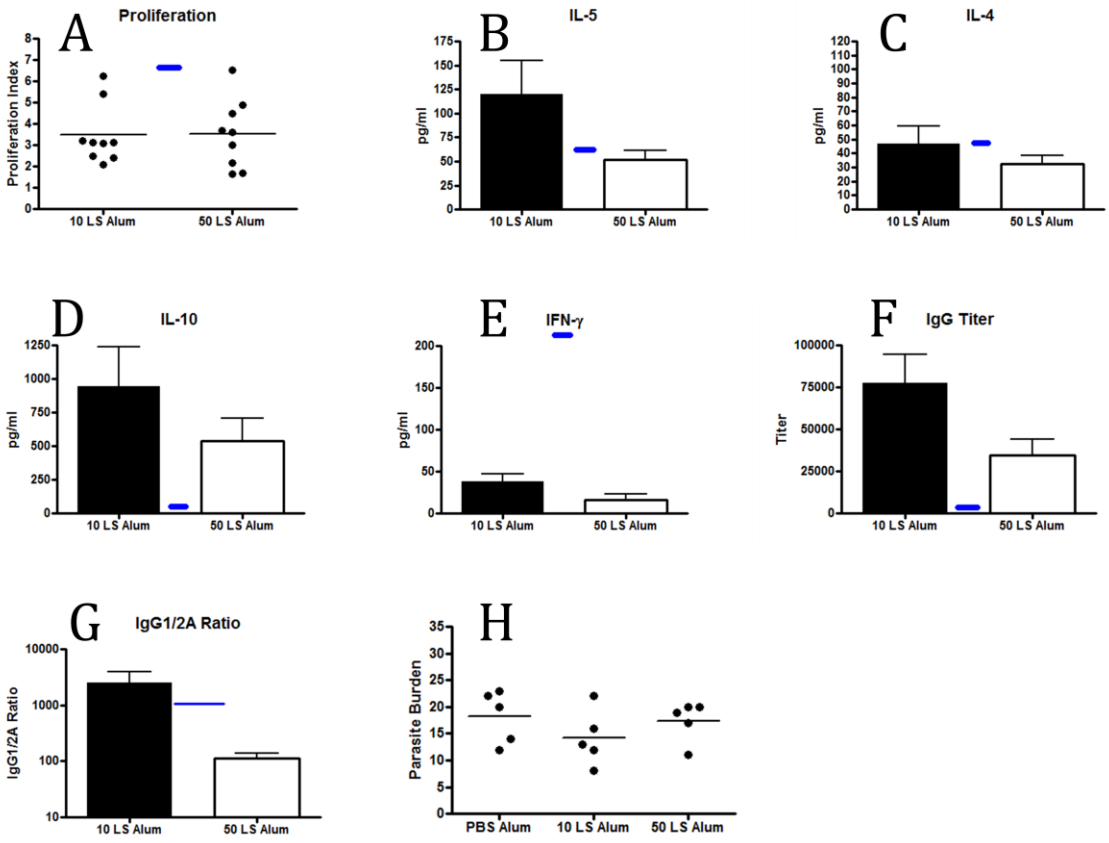


Figure 10: Immunological and protective responses in BALB/c mice vaccinated three times with 10 or 50 µg of LsAg plus alum (10 LS alum and 50 LS alum). A) Splenocyte proliferation in response to LsAg after 3 days of culture as measured by incorporation of BRDU. B) IL-5, C) IL-4, D) IL-10, and E) IFNγ concentrations above media stimulation in supernatants of splenocytes cultured for 3 days with 15 µg/ml of LsAg. F) LsAg-specific IgG titers and G) LsAg-specific IgG1 /IgG2a titers in plasma of mice vaccinated with 10 or 50 µg of LsAg plus alum. H) Parasite burdens 8 weeks after challenge infection in mice vaccinated with PBS plus alum (PBS alum), 10 µg of LsAg with alum (10 LS alum) or 50 µg of LsAg with alum (50 LS alum). Results for A-G are combined totals of two independent experiments. H) Parasite burdens in mice vaccinated with 10 or 50 µg LsAg using Alum as an adjuvant, or PBS vaccinated control mice. Results in H are from a single experiment using 5 mice for each group.

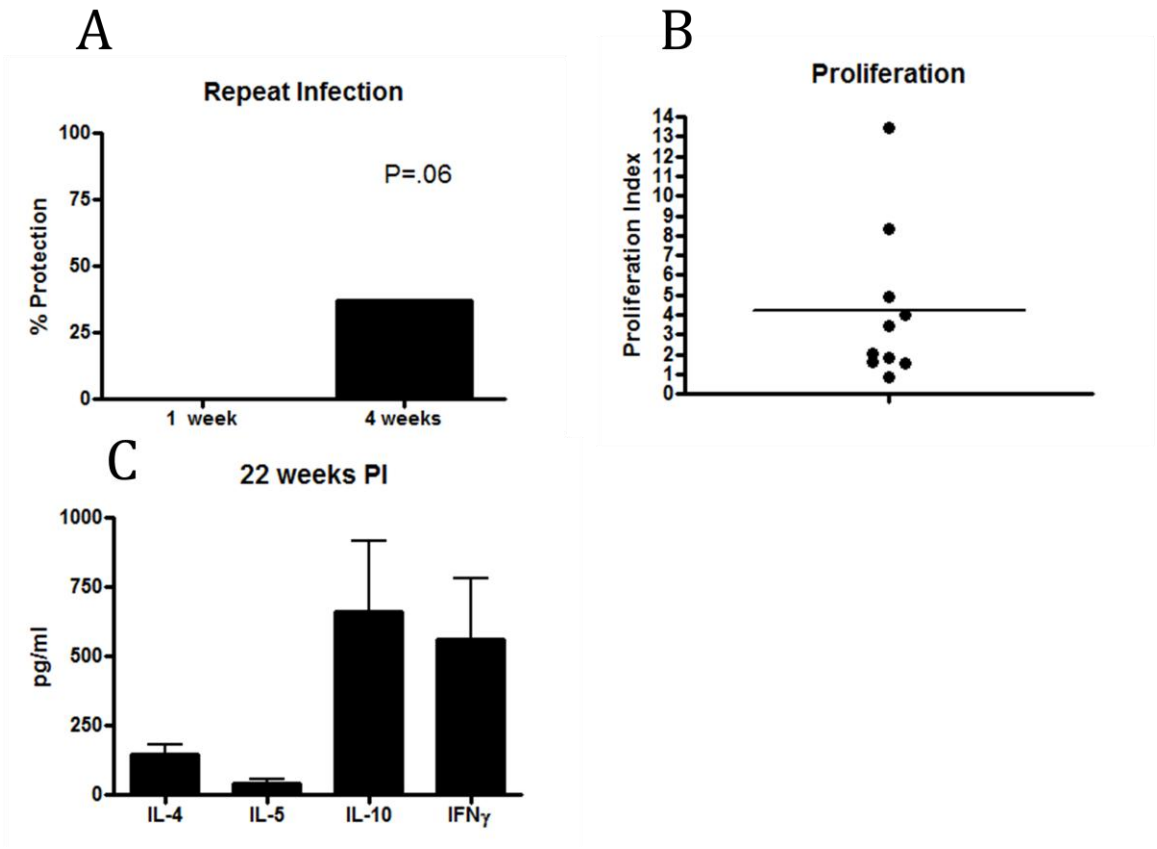


Figure 11: Immunological and protective responses in BALB/c mice after clearance of initial L. sigmodontis infection. A) Protection against repeat *L. sigmodontis* challenge in BALB/c mice 1 and 4 weeks after reinfection with 40 L3s. Re-infection was done at least 22 weeks after initial infection, and parasite burdens were compared to age matched controls receiving initial infection. B-C) Responses of splenocytes from BALB/c mice 22 weeks after initial infection when stimulated for 3 days with 15 μg/ml of LsAg. B) Proliferation of mouse splenocytes 22 weeks after infection in response to LsAg as measured by incorporation of BRDU. C) Cytokine concentration (above media stimulation) in supernatant of splenocyte culture media after stimulation for 3 days with 15μg/ml of LsAg.

Antibodies may be sufficient to induce modest protection, but are not necessary for protection after iL3 vaccination.

Data from other animal models of filariasis suggests that the protection garnered from both iL3 vaccination and repeat infection are due to immune responses to specific worm antigens (86; 101; 129). Western blot analysis, or LC/MS-MS of antigens that are bound by host antibodies, are powerful tools that could be used to determine which antigens are recognized by protected compared to non-protected mice. However, this type of analysis would be useful only if antibodies play a major role in worm clearance. We used two methods to determine the role of antibodies in protection against filariasis. In order to examine the sufficiency of antibodies to protect against challenge infection, we first incubated L3s for challenge infection in RPMI, plasma from previously infected animals, or plasma from iL3 vaccinated animals. This allowed antibodies from these mice to bind to the L3s. Next, we infected naive mice with these pre-incubated L3s, and determined parasite burdens 1 week later. The worms incubated in plasma from iL3 vaccinated mice showed a slight (28%), but significant, reduction in ability to survive compared to the RPMI incubated L3s (Figure 12A). To determine necessity of antibodies to induce protection after iL3 vaccination, we used the antibody deficient J_H mice. First, we verified that parasite burdens were similar during a primary infection of J_H mice compared to BALB/c mice (Figure 12B). Afterward, we vaccinated J_H mice and determined levels of protection 4 and 7 weeks PI. iL3 vaccinated JH mice showed 41% and 68% protection compared to controls 4 and 7 weeks PI respectively (Figure 12 C and D). While these protection levels are not as high as what is shown in BALB/c mice at the

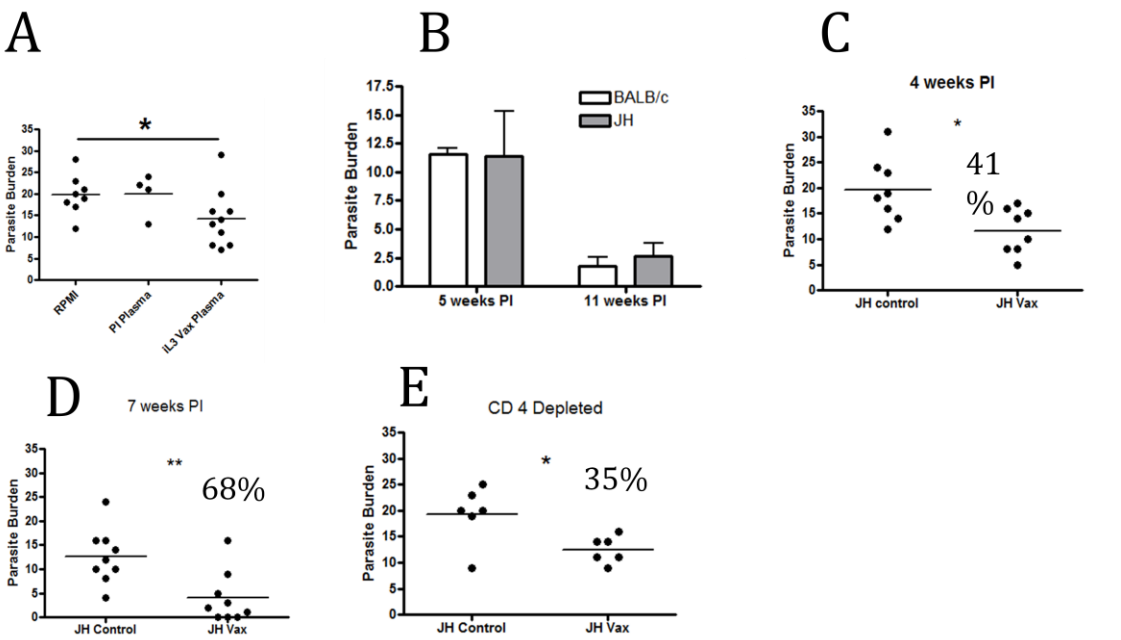


Figure 12: A) Survival of worms 1 week in BALB/c mice after 2 hour pre-incubation in RPMI, plasma from 22 week infected mice, or plasma from iL3 vaccinated mice. B) Parasite burden in a primary infection of BALB/c and JH mice. C-D) Worm burdens in antibody deficient JH mice vaccinated with iL3 and control mice 4 and 7 weeks post infection. E) Parasite burdens in iL3 vaccinated and control mice 4 weeks PI, wherein both vaccinated and control mice were depleted of CD 4 cells by injection of GK 1.5 2 days prior to and weekly throughout infection.

same time points (72% and 80%), they clearly show that antibodies are not necessary for a protective effect in this model.

CD4 cells are not necessary effector cells for vaccine induced protection.

Since clearance of worms was occurring within the pleural cavity as opposed to the peripheral tissues, it is likely that the events that lead to parasite clearance in vaccinated animals involve recruitment of immune cells to the site of infection. In mice infected with *Brugia* it has been shown that B and T cells are both important in the recruitment of cells to the site of infection. In order to more fully understand the role of $CD4^+$ T cells in the effector phase of this vaccine, we vaccinated J_H mice with irradiated larvae as before, and then depleted $CD4^+$ cells by administration of the GK1.5 antibody 2 days prior to infection and weekly until time of euthanasia. This resulted in 98% clearance of $CD4^+$ cells from the pleural cavity and nearly 100% from the blood (data not shown). Despite the lack of both mature B cells and $CD4^+$ cells at time of challenge and throughout infection, vaccinated mice still exhibited a moderate level of protection (35%) 4 weeks PI (Figure 12E).

DISCUSSION

In this paper we show a mechanism of protection after vaccination with irradiated larvae that is distinct from that shown in other labs. It has previously been shown that protection is directed against the L3 stage, occurs very quickly after challenge, and requires antibodies and IL-5(12; 83; 84; 96; 97). However, here we show that, with our protocol, the major parasite decline occurs 1-4 weeks PI and is not antibody dependent. While the number of irradiated larvae, number of vaccinations, time between vaccinations, and dose of irradiation given to larvae in this study matches those published in other labs, the source of larvae is different. In this study, we have used larvae that

have undergone some development within the jird, whereas other labs have used mite derived L3s. Some data from other filarial models suggest that the antigenic makeup of our L3s may be different from mite derived L3s. First, in the *A. viteae*/jird model, there is a change in the ability of peritoneal exudate cells from immune animals to bind to larvae after these have undergone some development within their definitive host (3). Similarly, transmission of *A. viteae* from the tick to the jird results in a rapid change in surface proteins on the infective larvae (8). This information suggests that vaccination with jird derived L3s may be distinct from vaccination with mite derived L3s. The ramifications of this are exciting, as this allows for a new effective vaccine regimen (vaccination with Late stage L3s) that can be emulated in future vaccine research. Similarly, this vaccine targets a different point of worm development, and perhaps a combination of these two vaccine approaches would allow for targeting challenge worms at two distinct points of development.

Apart from these somewhat unexpected findings, the main crux of this study was an attempt to use available knowledge of immune responses after vaccination with irradiated larvae in order to develop a vaccine approach that would be protective using soluble worm antigens, yet this approach was unsuccessful. Despite using three different vaccination protocols, and obtaining differential skewing of immune responses, none of these vaccination strategies were protective against future infection. In our first attempt, we utilized an adjuvant containing a combination of alum and CpG in order to stimulate both type 1 and type 2 immune responses. This was successful in stimulating both type 1 and type 2 associated antibody production, but Type 2 cytokine production in mice vaccinated with this regimen was very limited. In order to determine if Type 2 cytokine

responses to worm antigens would increase worm clearance, we attempted vaccinating using higher amounts of Alum as an adjuvant. This again failed to protect.

There are still several plausible explanations for the efficacy of the irradiated larval vaccine but not the LsAg vaccine. These include cytokine responses, differences in antigens present, and the site of immune responses after vaccination. For cytokine responses, although the LsAg Alum vaccine protocol induced type 2 cytokine production, this strategy also induced very high IL-10 responses, and overproduction of IL-10 has been linked to an abrogation of protection in resistant strains of mice (133). Thus one possible explanation for the failed protective immune response after LsAg/Alum vaccination is an improper ratio of pro-inflammatory to anti-inflammatory cytokines. Furthermore, vaccination with LsAg using neither Alum nor CpG/Alum as adjuvant resulted in a mixture of type 1 and type 2 cytokine responses, and protection after vaccination with jird derived iL3 may require both type 1 and type 2 responses. IL-4, IL-5 and IFNγ have all been shown to play roles in protection from *L. sigmodontis* (127; 153), and it is has been suggested that type 1 and type 2 cytokines have complementary effects against this worm (126).

One of the other possible explanations for the difference in protection between the LsAg vaccines and the irradiated larval vaccine is the difference in antigens present in each vaccine. Clearly the antigens present are from different life stages of the parasite. A recent proteomics analysis of *B. malayi* showed 292 proteins present in L3s that were not present in adults or MF (19). It is well known that vaccination with either abundant larval antigen I or II, which are specific to the larval stages, is protective in *B. malayi*(7; 55; 146; 149). Despite this, the finding that the majority of clearance occurs greater than

1 week post challenge infection suggests that this vaccination protocol is not targeting an L3 specific antigen. Instead, the presence of an insoluble protein that is targeted after irradiated larval vaccination is also possible. Indeed, in albino rats vaccinated against *L. sigmodontis* it was shown that vaccination with the soluble fraction of adult worms provided insignificant protection, yet vaccination with a homogenate of adult worms elicited 64% protection (100).

Finally, the third major explanation for the differential protection garnered after these vaccination strategies is the location within the host of major immune responses. Irradiated larvae localize to the pleural cavity. The clearance of these defective worms and their carcasses causes an infiltration of immune cells to the pleural space. It is unclear whether this results in any long-term changes in the presence of immune cells within the pleural cavity of mice, but challenge infection with our protocol always occurs 2 weeks post final vaccination. At this time, there is still an infiltrate within the pleural cavity, and the finding that $CD4^+$ cells are not required as effector cells suggests that there are still armed innate cells within the pleural cavity capable of killing off infective larvae. While it was shown that vaccination with mite derived iL3 elicits a long lasting protective response in mice (12), this has yet to be shown with jird derived iL3. Therefore, the possibility exists that the protective effect seen after this type of vaccination is simply the result of increased innate immune responses associated with the destruction of irradiated larvae.

While the repeat infection trial is a small part of this study, it is important for understanding the *L. sigmodontis*/BALB/c model of filariasis. At least 27 animal models of filariasis have been used to attempt vaccine research, and they show stark differences

in protection after repeat infection. A strong understanding of each model provides context and further understanding of any findings obtained in that model. Some models, such as *B. pahangi* in cats and *L sigmodontis* in cotton rats show little or no protection after repeat infection (42; 93). The *L. sigmodontis*/BALB/c model is powerful because of the availability of reagents to study protective mechanisms. However, while this model is considered permissive to infection, mice do eventually clear worms in an immune dependent manner (127; 153). This study suggests that possible protection garnered through repeat infections is moderate. This helps validate the use of this model in vaccine research.

In conclusion, this study highlights the complexity of host parasite interactions in filariasis. Very slight changes in any given protocol may completely alter the underlying host/parasite interactions. Despite this, our results suggest that there are multiple mechanisms that can be employed by the host to aid in protection against filarial worms. Recent results from other labs have suggested that using multiple target vaccines can in some cases increase protective results, and we suggest that further investigation of vaccination with both mite derived and jird derived irradiated larvae may result in further methods of protection against filariasis.

Chapter 4 A proteomic analysis of the body wall, digestive tract, and reproductive tract of *B. malayi*

INTRODUCTION:

Wuchereria bancrofti and *Brugia malayi* are infectious helminths that cause severe suffering in humans. Currently, there is a global effort, the GPELF, to eradicate lymphatic filariasis. While it is making good strides, efforts are limited by the need to repeatedly treat entire populations for 6-10 years (63; 116). The advent of new tools, such as vaccines or more effective anthelmintics, would be greatly beneficial to eradication efforts against these and other filariae.

The rational design of new tools against these terrible diseases, however, requires a strong understanding of the parasite's biology. Recent work in the field of genomics and proteomics has started to overcome our knowledge gaps (19; 20; 50). The genome of *Brugia malayi* has been published, and work is ongoing to discover the genome of *Wuchereria bancrofti*. Furthermore, studies on the proteins present in life cycle stages and excretory secretory (ES) products of *Brugia malayi* have been carried out. Similarly, key proteins in the reproductive processes of *B. malayi* have been identified (68; 69; 87). To date, no inclusive study has yet been done on the anatomic localization of proteins in filarial worms. Filariae are nematodes, parasitic metazoan creatures within the animal kingdom. As such, their anatomy is complex and includes body wall structures (cuticle, epidermis, musculature and lateral cords) as well as fully formed reproductive and digestive tracts (Fig 13). Knowledge of anatomic location of proteins within these parasites may provide information about likely physiologic function, and insight regarding potential rational approaches for drug and vaccine design.

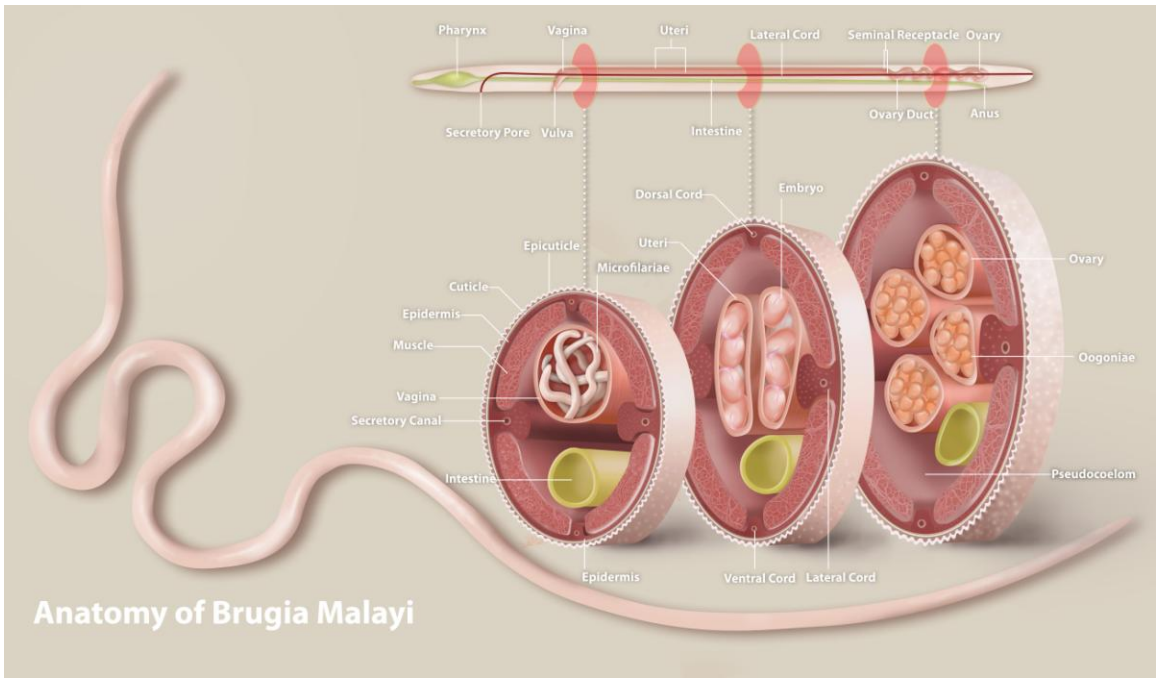


Figure 13: Anatomy of adult female *B. malayi*

One area of increasing interest in helminth parasitology is the use of intestinal antigens as vaccine candidates. The advantage to this approach is a lack of strong immune responses to these antigens in previously exposed hosts, thus providing novel methods of inhibiting the parasite's life cycle. Prior work with a number of helminths has shown potential for intestinal antigens as vaccines (table 4). Consequently, in addition to furthering our general understanding of the biology of filarial nematodes, our study also was done to identify unique surface expressed intestinal antigens of filariae for identification of possible vaccine candidates.

METHODS:

Dissections:

Dissections of approximately 500 previously frozen adult female *Brugia malayi* were carried out with a dissecting microscope and fine tipped forceps. One set of forceps was used to grip and steady the center of the parasite. Another set of forceps was used to grasp and gently twist the parasite close to the first set of forceps, resulting in a tear of the body wall. The cephalic tip of the body wall was then grasped and gently peeled away from the rest of the organs. The caudal portion of the body wall was then peeled away from the intestines and uterine tubes (Figure 14). Reproductive organs were identified by their anterior junction and then separated from the digestive tract. Each anatomic fraction (intestinal tract, reproductive tract, and body wall) was placed in a microcentrifuge tube of PBS. These were stored at -20°C until protein extraction.

Protein Extraction:

The samples were thawed and then centrifuged in 1.5 ml eppendorf tubes. The pelleted tissues were frozen and thawed 4 times by cycling through placement on dry ice for 10 min. followed by placement in a 37°C water bath. Using a mini disposable pestle, the samples were homogenized with 50ul of UPX extraction buffer (Expedeon).

Table 4: Protective intestinal antigen vaccines in various models of parasitic helminths

Aminopeptidase H11	90% (egg production)		*H. contortus*	Sheep	(164)
Arginase	56%	FCA	*S. japonicum*	Mice	(88)
Asparaginyl endopeptidase (Sm32)	37% less eggs	DNA vaccine	*S. mansoni*	Mice	(28; 30)
Sj32	38%	FCA	*S. japonicum*	Mice	(26)
Aspartic protease NaAPR1	66.6% egg reduction	CpG, Alhydrogel	*A. caninum*	Beagles	(112; 113)
Cathepsin L (FheCL1)	38-54%		*F. hepatica*	Cattle	(38)
Cathepsin L (FheCL2)	33-34%		*F. hepatica*	Sheep	(38)
Cysteine protease (*Ac*-CP2)	~65%	Various	*A. caninum*	Dogs	(90)
Integral membrane glycoproteins	~75%	QuilA	*H. contortus*	Sheep	(18)
M17 leucine aminopeptidase	49-87%	Various	*F. hepatica*	Sheep	(4; 94)

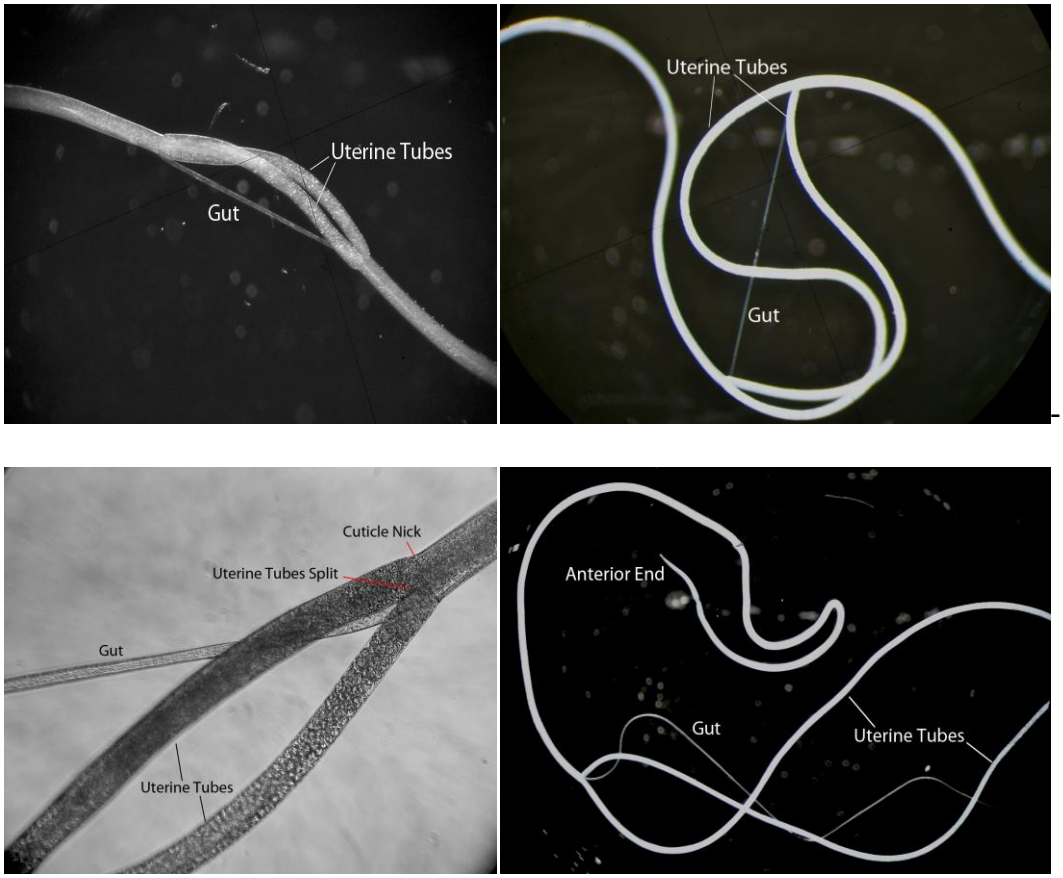


Figure 14: Dissection process of adult female *B. malayi.* Top left and bottom left show break in the body wall and extrusion of gut and uterine tubes. Top right and bottom right: Body wall is in process of being slid away from gut and uterine tubes.

The pestle was washed off with 50ul of UPX extraction buffer and processed as per instructions. In brief, samples were placed in a 100°C water bath for 5 minutes, removed and cooled at 4°C for one hour. Samples were then centrifuged at 15,000 x g for 10 minutes, and supernatant was collected. Proteins were desalted and digested with trypsin. This was followed by strong cat-ion exchange liquid chromatography fractionation.

RPLC-MS/MS:

400μg of protein from each sample was analyzed by nanopore RPLC-MS/MS with an Agilent 1100 Nanoflow LC system coupled online with a linear ion trap-Fourier transform mass spectrometer.

Protein identification:

Data obtained by LC-MS/MS was analyzed with SEQUEST against the Bm database obtained from the Institute for Genomic Research and against the *Wolbachia* database from New England Biolabs as described previously (19). Positive identification was defined as a tryptic peptide with 2 or fewer missed cleavage sites meeting specific criteria (delta correlation ≥ 0.08 and charge state-dependent cross-correlation scores ≥ 1.9 for [M+H]1+, ≥ 2.2 for [M+2H]2+, and ≥ 3.1 for [M+3H]3+). Dynamic modifications consisted of methionine oxidation and phosphorylation of serine, threonine, and tyrosine. Positive identification of a protein within this study was defined by the presence of at least 2 unique peptides within that anatomic fraction.

Enrichment analysis:

We analyzed proteins based on the number of unique peptides found within each fraction that matched to a particular protein. The terms enriched and highly enriched will be found throughout the paper. For the purpose of this paper, enriched is defined as

$\frac{\text{\#Peptides in target fraction}}{\text{\#peptides in other two fractions combined}} > 1$, and highly enriched is defined as $\frac{\text{\#Peptides in target fraction}}{\text{\#peptides in other two fractions combined}} \geq 2$.

Quantitative analysis:

The assigned spectra for each protein were analyzed with a normalization scheme to provide an arbitrary gauge of relative quantitation termed Quantitative value. The quantitative values of individual proteins were compared within a specific anatomic fraction that was run in order to provide a hierarchy of quantity within that fraction. Quantitative values of any protein were not compared between anatomic fractions.

Functional analysis:

The functional proteome of *B. malayi* had previously been performed by Bennuru and colleagues (19). For proteins previously annotated for function, no further analysis of function was carried out. The 665 newly identified proteins were annotated based loosely on the KOG and PFAM functions. Categories of function were used as previously described (19), including cytoskeletal, extracellular matrix, immunological, metabolism, nuclear regulation, protein export, protein modification, protein synthesis, signal transduction, transcription, transporters, and uncharacterized. Analyses of the functional profile of each organ were performed in two different ways. First, organ functional profile was graphed based solely on the percentage of proteins present within each organ associated with each function. Second, organ functional profiles were analyzed based on Gene Set Enrichment Analysis, which analyzes the data for bias in a condition (or anatomic fraction) (139). Proteins were ranked according to abundance. A priori defined sets of proteins, based on functional annotation, were then analyzed using GSEA for bias within each organ.

RESULTS:

Distinct anatomic fractions exhibit markedly different expression of proteins

Based on a match of 2 unique peptides to a protein, we identified a total of 5023 proteins. Of these, 204 were Wolbachia proteins, and 34 could be matched to more than 1 specific protein, leaving 4,785 specifically identified *B. malayi* proteins. 665 proteins had previously not been identified in *B. malayi* (Figure 15). 1,895 of the proteins were identified in all three organs of the parasite. However, 396 proteins were identified solely within the digestive tract, 114 were located solely within the body wall, and 1011 were found solely within the uterine tubes. Additionally, although the majority of proteins were present in all three organs, we found that there was a highly differential expression of each protein among the anatomic fractions (Fig. 16).

Validation of dissection accuracy

To gauge the accuracy of this study, we first analyzed the location of microfilarial sheath proteins, which logic suggests would only be found within the uterine tubes of adult female worms. Our study found 4 microfilarial sheath proteins. While peptides from each were typically found in all 3 anatomic fractions, quantitative analysis demonstrated high enrichment of microfilarial proteins within the uterine tube fraction (See figure 17A). The finding of low levels of microfilaria proteins in gut and body wall suggest some level of contamination between the anatomic fractions, which was expected given the challenge of manually dissecting filarial worms.

In another method of examining the accuracy of this study, we evaluated our results for proteins that had previously been identified within microfilariae obtained from uterine tracts of adult female worms (UTMF) and that were not present within adult male worms. In that study, 989 proteins were found that had at least 2 unique peptides in the UTMF, but no peptides within the adult male (19). We identified 645 of these

5023 Proteins
204 Wolbachia Proteins Minimum 2 peptides
34 Proteins too similar to differentiate
4785 Uniquely identified Brugia Proteins

NEWLY IDENTIFIED PROTEINS

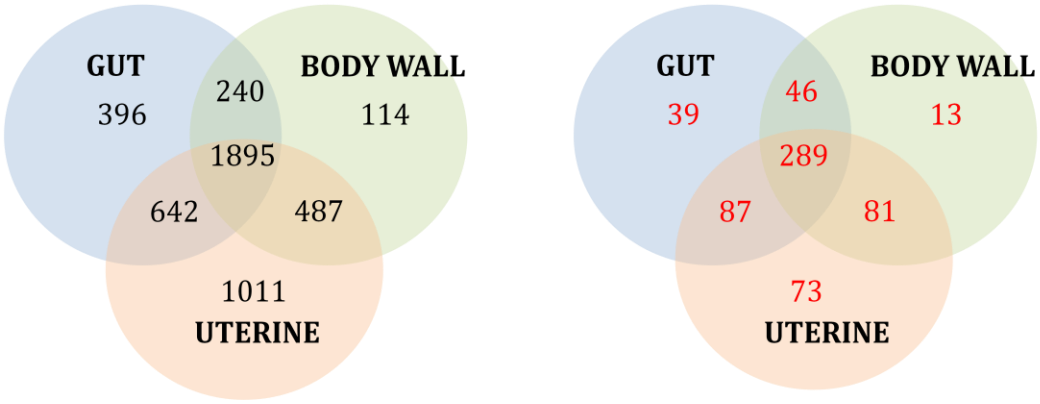


Figure 15: Venn diagram of proteins identified within each anatomic fraction of adult female *Brugia malayi*

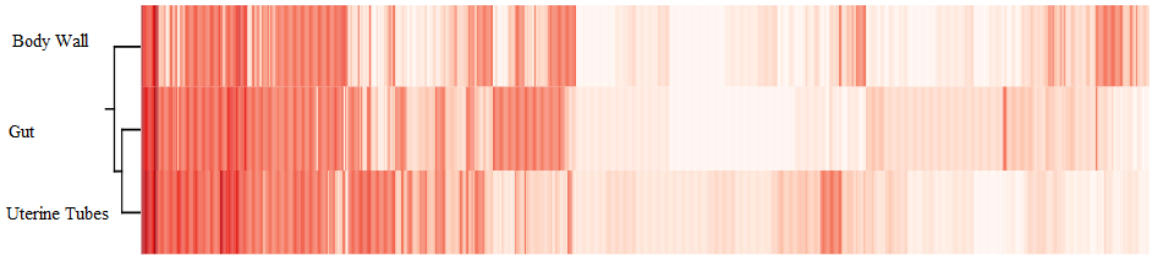


Figure 16: Heat map of proteins found within the different anatomic fractions of adult female *B. malayi*

proteins within our study. As seen in Figure 15B, the vast majority of proteins that were enriched in one fraction were in the uterine tract. Indeed, 353 proteins of uterine tube microfilariae were found enriched (n=129) or exclusively (n=224) within the reproductive tract at that site (Figure 17 B). In contrast, only 58 of these proteins were specific to or enriched within the gut, and only 22 were specific to or enriched in the body wall.

Distinct anatomic fractions exhibit functional bias in protein expression

We conducted gene set enrichment analysis (GSEA) to determine whether the proteins present within each anatomic fraction have a bias towards particular functions. GSEA determines functional bias by ranking an *a priori* defined set of proteins within a particular fraction by their quantitative value and comparing the presence of these proteins to the other fractions. As seen in figures 18-20 each anatomic fraction displayed a functional bias. Specifically, intestinal tract displayed bias for transporter function, body wall exhibited bias for cytoskeletal proteins and proteins of immunological interest, and the reproductive tract had a marked bias for proteins associated with transcription and nuclear regulation.

Proteomic profiling of the *B. malayi* intestine is consistent with functional absorption and digestion

Like all nematodes, filarial parasites have a fully formed intestinal tract. However, the functionality of this tract is not completely clear, as several studies have suggested that much of the necessary nutrition for *B. malayi* can be obtained by absorption through the cuticle (108). In order to gain a better understanding of the function of the digestive tract of *B. malayi*, we sorted the proteins that were highly

enriched within the gut based on their quantitative abundance (Table 5). Of the 20 most abundant highly enriched gut proteins, 6 are transporters, 2 are proteolytic

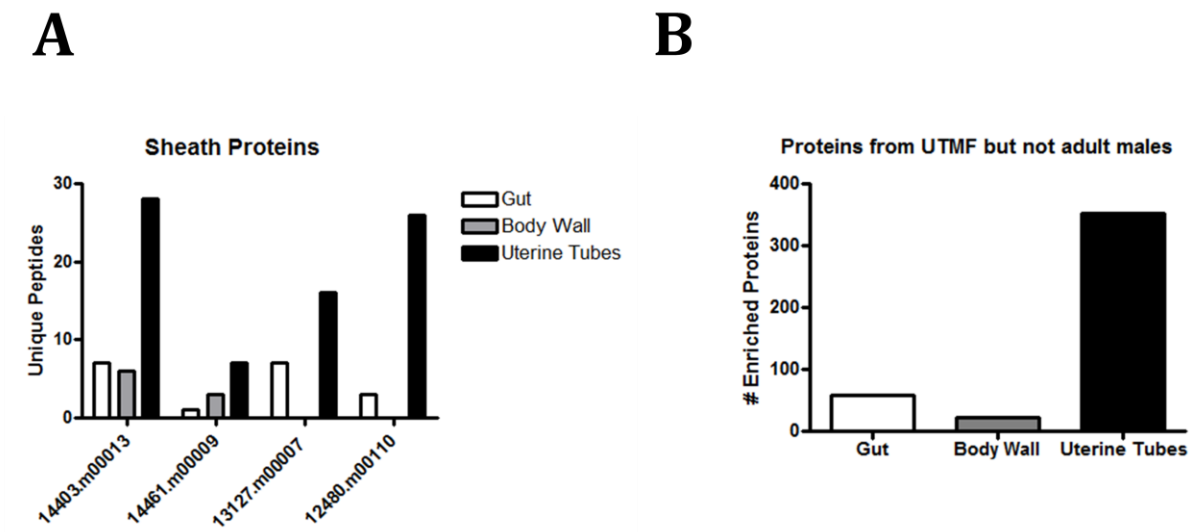


Figure 17: A) Number of unique peptides from each of the 4 microfilarial sheath proteins in each anatomic fraction. B) Enrichment of proteins previously identified in microfilariae obtained from the uterine tubes, but not identified within adult males, within each anatomic fraction.

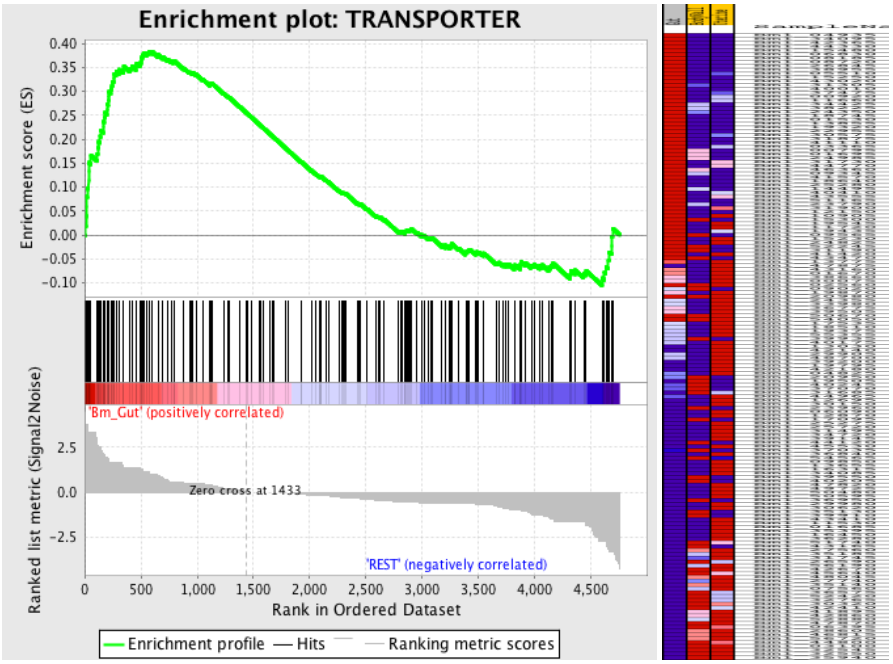


Figure 18: Association of transporter proteins with the digestive tract as measured by gene set enrichment analysis (GSEA). P-value=0.005 The enrichment score is represented by the green line. Proteins were rank ordered according to their abundance within the gut, and are depicted in the heat map (red= more abundant, blue= less abundant). Black vertical lines represent each of the proteins associated with transporter function.

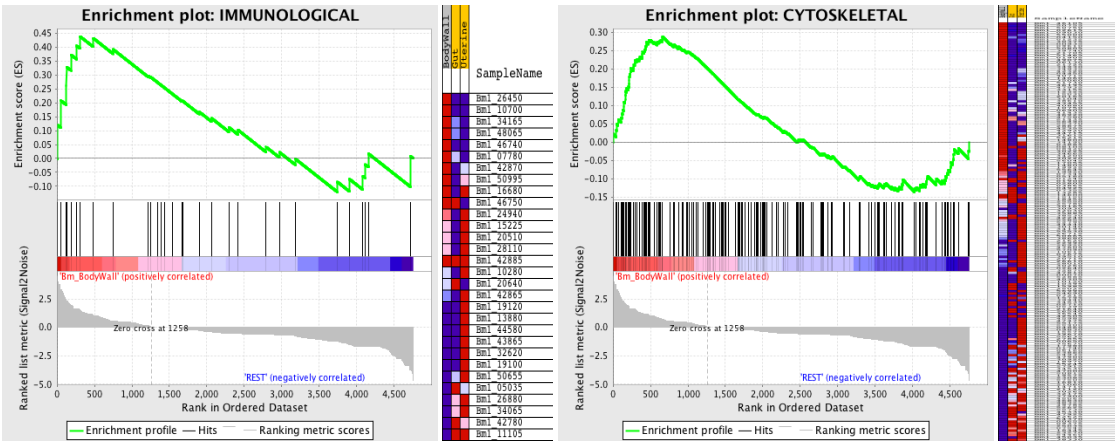


Figure 19: Association of Immunological and Cytoskeletal proteins with the body wall of the adult female *Brugia malayi* as measured by GSEA. P-value=0.003 and 0.009 respectively. The enrichment score is represented by the green lines. Proteins were rank ordered according to their abundance within the body wall, and are depicted in the heat map (red= more abundant, blue= less abundant). Black vertical lines represent each of the proteins associated with proteins of immunological interest (left) and cytoskeletal proteins (right) function.

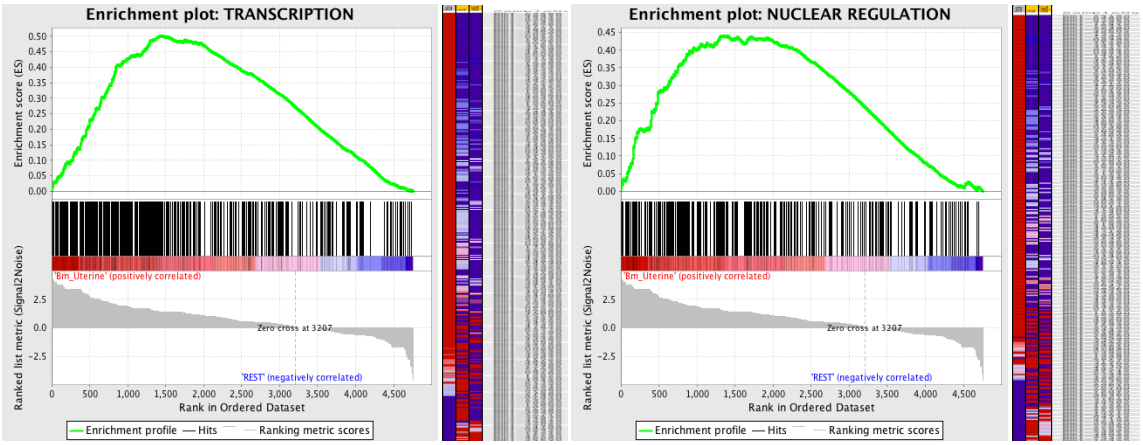


Figure 20: Association of transcription (P<0.001) and nuclear regulation proteins (P=0.013) with the reproductive tract of adult female *B. malayi*. The enrichment score is represented by the green line. Proteins were rank ordered according to their abundance within the uterine tubes, and are depicted in the heat map (red= more abundant, blue= less abundant). Black vertical lines represent each of the proteins associated with transcription (left) and nuclear regulation (right).

enzymes, 2 are involved in lipid metabolism, 1 is involved in glucuronidation, and 1 is a histidine acid phosphatase. Based on this information, and the GSEA analysis, it is likely that the gut of *B. malayi* is involved in digestion and absorption. Both of the identified proteolytic enzymes are predicted to be secreted suggesting that their action would be within the lumen of the intestines and not intracellular. However, one of the most abundant gut enriched proteins was UDP-glucoronosyl and UDP-glucosyl transferase which is associated with phase II metabolism, suggesting that another function of the gut may be to remove xenobiotics from the worm.

An examination of the most abundant proteins found within the gut of the worm also provides some further insight into the function of the gut (Supplementary table 1). The most abundant protein found within the gut of *B. malayi* was a short chain dehydrogenase/reductase. Short chain dehydrogenases have been shown to be important in the metabolism of sugars, alcohols, prostaglandins, other lipids and xenobiotics (72; 102). Short chain dehydrogenase/reductases have been previously mentioned as possible drug targets. Other protein types that were present in high levels in the gut were heat shock proteins, cytoskeletal proteins, lectins, enzymes associated with glycolysis, an elongation factor, and a precursor to collagen IV which makes up the basement membrane. Of these proteins, the two lectins, which are more specifically galectins, deserve special mention, as they have been associated with many various functions within nematodes including cell to cell binding and modulation of host immune responses.

Table 5: 20 most abundant highly enriched proteins in the gut of adult female *B. malayi*

Gut Enriched Proteins	# Unique Peptides		
	Gut	Body wall	Uterine Tube
Transporters			
TIGR_13617_m00049 AMOP domain containing protein 1513 bp	46	2	14
TIGR_14947_m01132 Amino acid permease family protein 862 bp	16	3	1
TIGR_14731_m00991 Major Facilitator Superfamily protein 493 bp	8	0	2
TIGR_14972_m07707 NRAMP-like transporter K11G12_4, putative 496 bp	7	0	2
TIGR_14962_m00670 Ctr copper transporter family protein 276 bp	3	1	0
TIGR_14947_m01131 Na+/K+/2Cl- cotransporter-like 374 bp	4	1	0
Miscellaneous Function and Hypothetical Proteins			
TIGR_14952_m01392 hypothetical protein 222 bp	8	0	2
TIGR_14972_m07788 hypothetical protein 1209 bp	4	0	1
TIGR_14979_m04435 Mospd2 protein, putative 558 bp	8	1	1
TIGR_14561_m00009 Acyl CoA binding protein 115 bp	6	2	0
TIGR_14025_m00013 hypothetical protein 217 bp	4	0	0
TIGR_14652_m00411 Hypothetical protein 314 bp	2	1	0
TIGR_12665_m00134 MGC69076 protein-related 129 bp	7	1	1
TIGR_14187_m00209 Acyltransferase family protein 649 bp	5	1	1
Proteolytic Enzymes			
TIGR_14963_m01819 aspartic protease BmAsp-1, identical 393 bp	8	3	0
TIGR_14387_m00341 Papain family cysteine protease containing protein 314 bp	8	0	0
Lipid Metabolism			
TIGR_13362_m00015 NAD-dependent malic enzyme, mitochondrial precursor, putative 425 bp	11	3	1
TIGR_15062_m00045 NAD-dependent malic enzyme, mitochondrial precursor, putative 555 bp	9	1	2
Glucuronidaton			
TIGR_14006_m00103 UDP-glucoronosyl and UDP-glucosyl transferase family protein 538 bp	19	2	0
Hydrolases			
TIGR_14731_m00987 Histidine acid phosphatase family protein 411 bp	12	3	1

Predominant body wall proteins have structural, kinetic, and energy metabolism functions

The body wall of *B. malayi* includes, from superficial to deep, the epicuticle, cuticle, epidermis, musculature and the lateral cords (See figure 13). The musculature is separated into quadrants by the lateral, ventral and dorsal cords with up to 9 myocytes per quadrant (108). The lateral cords contain the cell bodies of the epidermis which produces and maintains the cuticle. Also associated with the lateral cords is a secretory gland which is connected to the secretory pore by the secretory canal (81). The ventral and dorsal cords are associated with nerves that innervate the musculature.

Of the 20 most abundant proteins in the body wall of *B. malayi*, 13 of them were, as expected, cytoskeletal proteins involved in muscle structure (See supplementary table 2). Protein UNC-22, which is involved in muscle contraction, was also highly abundant. There were also 3 proteins involved in glycolysis, a galectin, and the short chain dehydrogenase/reductase that was the most abundant protein in the gut. Additionally, carbohydrate phosphorylase, which is an enzyme that catabolizes glycogen, was both enriched within the body wall and highly abundant. The myocytes within nematodes have an exterior region associated with contraction, and a separate region of the cell that stores and metabolizes glycogen. Although it was not abundant enough to be in the top 20 list, glycogen synthase was also enriched within the body wall.

An analysis of the most abundant proteins that were highly enriched within the body wall provides very similar results (See table 6). Again many of these proteins were associated with muscle structure or regulation of muscular contraction. There were also several cell adhesion molecules, including 2 immunoglobulin-i domains, and 3 proteins that aid in energy production. 1 of these proteins, the amylo-alpha-1,6-

Table 6:20 most abundant highly enriched proteins in the body wall of adult female *Brugia malayi*

	#Unique Peptides		
Body Wall Enriched Proteins	Gut	Body Wall	Uterine Tube
Cytoskeletal/muscular			
TIGR_14972_m07587 protein unc-22, putative 6781 bp	49	268	29
TIGR_15274_m00010 kettin, putative 1319 bp	13	64	19
TIGR_14848_m00007 Prion-like-, putative 626 bp	6	27	5
TIGR_12711_m00019 Paramyosin, identical 44 bp	2	10	1
TIGR_15558_m00007 Prion-like-, putative 360 bp	4	16	4
Cell Adhesion			
TIGR_13877_m00097 Immunoglobulin I-set domain containing protein 4791 bp	15	148	23
TIGR_14081_m00291 Immunoglobulin I-set domain containing protein 9444 bp	22	103	22
TIGR_14972_m07232 LIM domain containing protein 636 bp	3	19	5
Miscellaneous			
TIGR_14972_m07915 predicted protein 928 bp	10	36	8
TIGR_14396_m00009 conserved hypothetical protein 762 bp	6	38	4
TIGR_12897_m00018 hypothetical protein 474 bp	9	27	3
TIGR_14972_m07082 conserved hypothetical protein 154 bp	3	18	3
TIGR_14237_m00394 Hypothetical protein-conserved 411 bp	0	17	7
TIGR_14972_m07518 Animal haem peroxidase family protein 999 bp	7	21	2
Energy Metabolism			
TIGR_14972_m07100 Sugar transporter family protein 595 bp	5	19	4
TIGR_14955_m00258 oxidoreductase, aldo/keto reductase family protein 317 bp	9	28	4
TIGR_14393_m00060 Amylo-alpha-1,6-glucosidase family protein 499 bp	6	18	2
Neuromuscular Protein			
TIGR_14990_m07885 EF hand family protein 161 bp	2	18	7
TIGR_14979_m04538 Ryanodine Receptor TM 4-6 family protein 5072 bp	9	70	17
Cuticle			
TIGR_14972_m07115 Cuticle collagen 13 precursor, putative 329 bp	2	8	2

glucosidase catabylizes glycogen. Somewhat surprisingly, only 1 of the proteins is a cuticle protein. This may simply be the result of cuticle proteins not being specific to the body wall. Of the 30 cuticular collagens that were identified within *B. malayi*, only 12 were either enriched or specific to the body wall (See supplementary figure 1). This is most likely because microfilariae contained within the uterine tubes contain a cuticle and portions of the digestive tract (stomadeum and proctodeum) are lined with a cuticle (85). Indeed, there were 7 cuticle proteins that were either enriched in or specific to the uterine tube. When these 7 UT-associated proteins were compared to previous results, 2 had not previously been identified, and the other 5 had previously been identified within MF or MF obtained from the UT of adult females (See supplementary figure 2A).

Highly enriched and abundant proteins within the female reproductive tract have various functions including interaction with nucleic acids and controlling the cell cycle.

The nematode female reproductive tract consists of 2 ovaries where gamete production takes place, 2 seminal receptacles (aka spermatheca) which store sperm obtained from males, and 2 uterine tubes that allow for embryo and subsequent MF development (Figure 13). The two uterine tubes merge into the vulva, which is on the ventral surface of the worm in the cephalic region (46; 69; 81; 87). Histologically, the majority of the reproductive tract is an endothelial lined tube containing gametes, embryos, and developing microfilariae. The most abundant group of proteins that were highly enriched within the female reproductive interact with nucleic acids such as histones, topoisomerases, and splicing factors (Table 7). Similarly, two different cell cycle proteins were enriched within the uterine tubes. In *B. malayi* worms, only the microfilaria stage has an outer acellular sheath. Consistent with this, two different sheath

Table 7: 20 most abundant highly enriched uterine tube proteins in adult female *B. malayi*

Uterine Tube Enriched	# Unique Peptides		
Nuclear reactive proteins	**Gut**	**Body Wall**	**Uterine Tubes**
TIGR_14500_m00158 histone H2A, putative 126 bp	1	0	2
TIGR_12698_m00326 histone H3, putative 136 bp	2	1	13
TIGR_12743_m00017 Histone H2B 2, putative 149 bp	0	0	5
TIGR_14981_m02406 Probable DNA topoisomerase II, putative 1570 bp	14	4	45
TIGR_14424_m00385 splicing factor 3B subunit 3, putative 1181 bp	7	4	22
Sheath Proteins			
TIGR_14403_m00013 Major microfilarial sheath protein precursor_-related 195 bp	7	6	28
TIGR_13127_m00007 sheath protein 5, identical 161 bp	7	0	16
Hypothetical proteins			
TIGR_13392_m00051 hypothetical protein 727 bp	11	3	35
TIGR_14979_m04589 hypothetical protein 207 bp	5	1	12
TIGR_14387_m00360 hypothetical protein, conserved 983 bp	11	0	30
TIGR_14704_m00449 hypothetical protein 627 bp	3	1	27
Miscellaneous			
TIGR_14569_m00224 Chitin binding Peritrophin-A domain containing protein 2488 bp	12	11	82
TIGR_14284_m00379 Fasciclin domain containing protein 623 bp	9	3	28
TIGR_14971_m02876 PAN domain containing protein 965 bp	4	5	24
Cell cycle/cell signaling			
TIGR_15557_m00013 retinoblastoma-binding protein_, putative 369 bp	11	2	27
TIGR_14646_m00062 Phosphatidylinositol 3- and 4-kinase family protein 2333 bp	11	5	33
Cytoskeletal			
TIGR_14990_m07700 Filamin/ABP280 repeat family protein 1719 bp	15	5	47
Xenobiotic metabolism			
TIGR_14961_m04939 Flavin-binding monooxygenase-like family protein 528 bp	13	3	33
Energy metabolism			
TIGR_14967_m01561 oxidoreductase, zinc-binding dehydrogenase family protein 2507 bp	5	7	42
Nuclear import Proteins			
TIGR_15544_m00028 Importin beta-1 subunit, putative 884 bp	8	5	29

proteins were found to be both highly enriched and highly abundant within the female reproductive tract.

Similar to the other anatomic fractions, many of the most abundant proteins within the female reproductive tract were cytoskeletal proteins or involved in glycolysis (Supplementary table 3). However, one major difference is that 4 histones were among the 20 most highly abundant proteins within the reproductive tract, while no histones were found within the 20 most highly abundant proteins of either of the other 2 anatomic fractions.

Trehalose metabolism is found in all three anatomic fractions

Trehalose is a disaccharide that is used for some energy storage in nematodes and is involved in various aspects of worm biology, such as hatching, stress protection, and maintaining osmotic pressure for the hydrostatic skeleton. As there are no homologs to the anabolic trehalose pathway in humans, this is a pathway that could be pharmacologically targeted (80). We found that trehalose-6-phosphate synthase was present in all 3 organs of the worm, and trehalose-6-phosphate synthase-related protein was enriched within the uterine tubes. While we identified trehalase family proteins within every organ of the worm, some were preferentially found in one anatomic fraction. Of the five different trehalase family proteins identified in this study, 14977.m05009 was specific to the uterine tubes, 14982.m02257 was enriched within the uterine tubes and 14813.m00044 was specific to the body wall (supplementary figure 2B).

Identification of potential gastrointestinal vaccine candidates

One of our goals in conducting this study was to identify potential novel vaccine candidates for filariasis. To identify gastrointestinal proteins that could potentially be

used as vaccine candidates, we analyzed our proteomics set for proteins that were both highly enriched in the intestinal tract, and either had a secretory signal or a trans-membrane domain. We sought surface or secreted proteins because these proteins are likely accessible to host antibodies after vaccination. 75 proteins matched these criteria and can be found in supplementary table 4.

19 of these proteins were transporters. Transporters have been used successfully as vaccine candidates in some models of bacterial diseases such as *Streptococcus pneumoniae* (23). Furthermore, the amino acid transporter SPRM1 has been suggested as a vaccine candidate in schistosomiasis (79). For an intestinal transporter to be effective as a vaccine antigen in filariasis, there would have to be the possibility of the antibody preventing the function of the transporter by binding to it, thus depriving the parasite of important nutrients. For this reason, we believe that active transporters with specific substrates may make effective vaccine candidates.

One other possible vaccine target is the oxysterol binding protein. Oxysterol binding proteins are similar to other lipid binding proteins, such as fatty acid binding protein, and may play a role in either cell signaling or oxysterol absorption. Fatty acid binding proteins have shown protection when used as vaccine candidates in schistosomiasis, and have even been cross protective against *F. hepatica* in sheep (145).

There were 5 proteolytic enzymes that were highly enriched within the gut and found to have transmembrane domains or were predicted to be secreted. There was also 1 serpin, a proteolytic enzyme inhibitor, which matched the criteria for a possible hidden antigen vaccine candidate. There were many other identified enzymes that could

potentially be targeted with either vaccination or drug therapy, and they can be found in supplementary table 4.

Certain ES products are associated with specific anatomic fractions

227 proteins were found within the ES product of adult female *B. malayi* in a previous study by Bennuru and colleagues (20). In order to better define the origin of these proteins, we analyzed all adult female ES proteins for enrichment within any of the 3 worm fractions from this study. 8 (3.5%) of these proteins were either enriched or specific to the gut (see supplemental table 5). The most notable of these was the papain family cysteine protease 14387.m00341. There was also the patched family protein, which is a receptor for sonic hedgehog, and fumarylacetoacetatehydrolase, an enzyme involved in tyrosine catabolism (136). 11 (4.8%) female ES products were enriched within the body wall (See supplementary table 6), including 2 proteins that protect against oxidative damage, cuticular glutathione peroxidase and peptide methionine sulfoxide reductase (156). Other ES products enriched within the body wall included many muscle proteins, an aminopeptidase, and a HAD hydrolase.

There were 49 adult female ES products (21%) enriched within the female reproductive tract (supplementary table 7). Some of these antigens include Juv-p120, two different trypsin inhibitors, and phosphatidylinositol 3- and 4- kinases.

Identification of protective vaccine candidates

In order to gain better understanding of previously effective vaccine candidates, we matched previously protective vaccine candidates from *B. malayi*, by amino acid sequence to the current naming system for *B. malayi* proteins. Only vaccine studies using *B. malayi* in a permissive model were considered for this analysis. Of the 10 specific *B. malayi* antigen vaccine candidates previously shown to induce protection in jirds against

infection with *B. malayi*, 6 were identified within the adult female worm within this study (see supplementary table 8). Both of the muscular proteins, myosin and paramyosin, were more associated with the body wall than the other 2 organs. Transglutaminase, thioredoxin peroxidase, glutathione-S-transferase and BmSL3 were also found in this study, and were represented similarly between the 3 organs. Vespid allergen homolog, superoxide dismutase, and the two abundant larval transcripts were not found in this study.

DISCUSSION

The purposes of this study were to try to determine vaccine targets that may be sequestered within the intestine of the parasitic helminth *B.* malayi, and to gain more information on the biology of this nematode. In order to accomplish this, adult female *B. malayi* were dissected into worm fractions containing the digestive tract, the reproductive tract, or the body wall. These fractions went through a protein extraction process, and proteins present were identified by RPLC-MS/MS. The validity of results was confirmed by analyzing enrichment of sheath proteins within the female reproductive tract, and by determining concordance with past studies. Furthermore, the presence of many muscle associated proteins within the body wall and many proteins with actions on nucleic acids within the reproductive tract are highly consistent with our understanding of *B. malayi*.

Because of strong evidence within the field that helminth digestive tract antigens may make effective vaccine candidates, we focused on this anatomic fraction for vaccine purposes. While a cursory understanding of the nematode digestive tract would suggest that the major role of the digestive tract in this nematode is nutrient digestion and absorption, several factors in the biology of *B. malayi* have led researchers to question the role of the digestive tract in this nematode. First, *B. malayi* lives within the lymphatics of

its host, and can directly ingest lymphatic fluid, which has a composition similar to plasma. Therefore, this pathogen has a rather low need to metabolically digest substances. Second, when food is not present within the digestive tract of nematodes, the high pressure in the pseudocoelom keeps the intestines collapsed, and H and E staining of a cross section of *B. malayi* routinely reveals a closed intestine. Third, analysis of the MF, L2 and L3 stages of the life cycle of *B. malayi* reveal a non-functional alimentary canal. Lastly, water, nucleotides, amino acids, small peptides, sugars and vitamins can be directly absorbed through the cuticle of *Brugia* (108). All of these data taken together suggest that the intestinal tract of *B. malayi* may play only a minor role in digestion and absorption. In contrast to this, analysis of the most abundant highly enriched gut proteins revealed proteins that, in many cases, are associated with digestion and absorption, which is consistent with the general notion that the intestine of *B. malayi* does engage in digestive and absorptive processes. Furthermore, GSEA analysis suggests an association between the gut and transporter proteins. While these two findings are not direct evidence for the role of the gut, they are consistent with the gut performing at least some of the digestion and absorption in adult female *B. malayi.* This validates the plausibility of inducing protective immune responses by vaccination with intestinal antigens in this model.

In order to identify potential vaccine candidates, we analyzed the data for trans-membrane or secreted proteins that were highly enriched or specific to the digestive tract. As almost all vaccines targeted against the intestine of helminths have been proteolytic enzymes, the 5 proteolytic enzymes found to be highly enriched within the gut are prime candidates for future research. The one major caveat to this is that the papain family

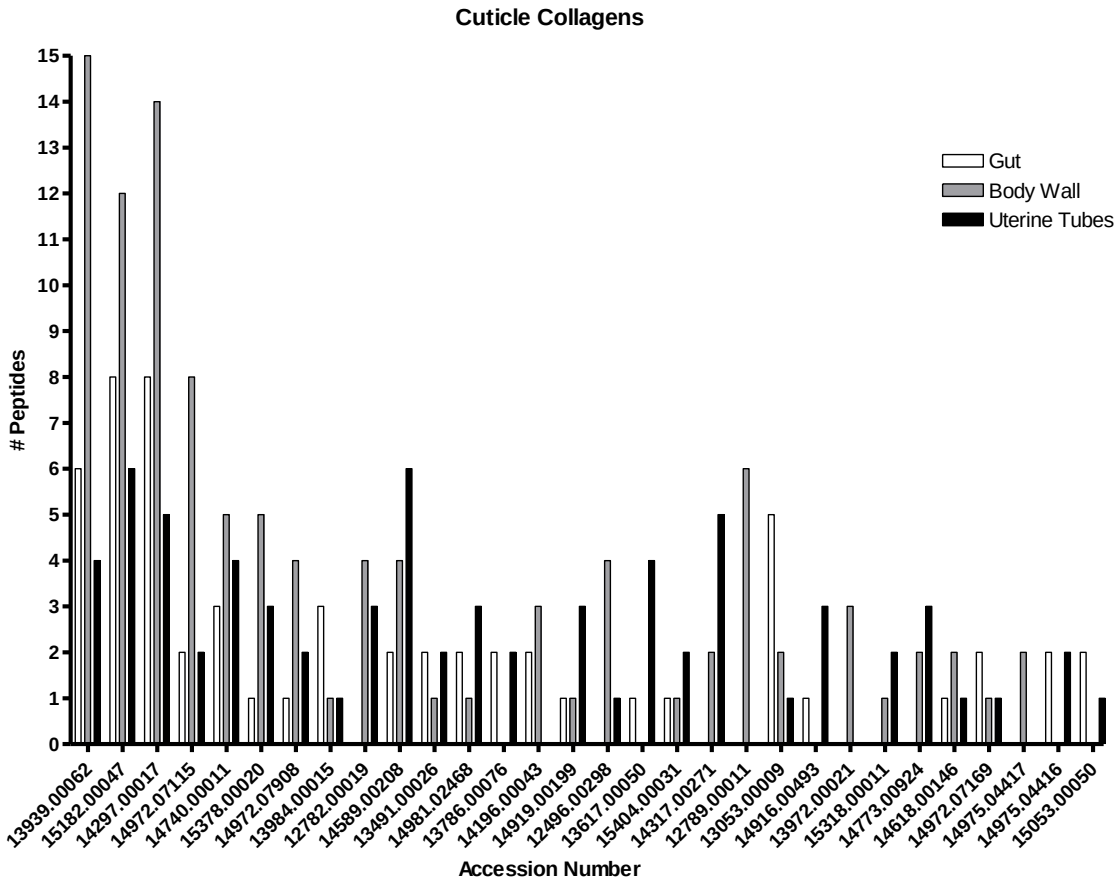
cysteine protease was previously identified within the ES product of adult female worms. This suggests that this specific proteolytic enzyme may still pose a threat of allergic reactions in exposed individuals.

While only one protease inhibitor was enriched within the intestine, it is important to mention this type of vaccine candidate because of prior work that has been done using protease inhibitors as vaccine candidates in filariasis. The cystatin and serpin protease inhibitors are thought to aid the worm by preventing host proteolytic enzymes from digesting the parasite (40; 163). It was previously hypothesized that these types of proteins would make good vaccine candidates. In a mouse model of onchocerciasis, vaccination with cystatin adsorbed to alum provided 34% protection (31). However, vaccination with helminth derived proteolytic inhibitors is not always protective (40; 106).

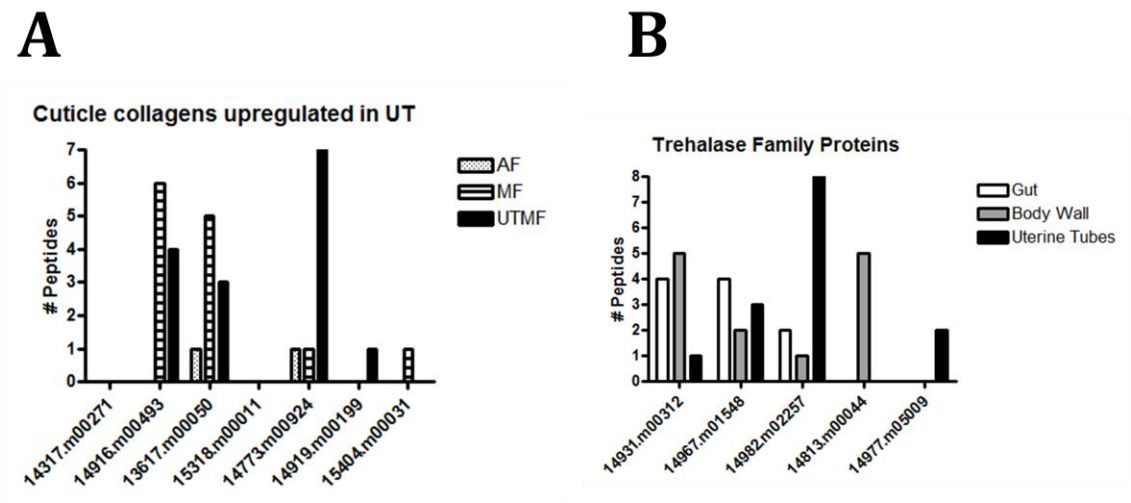
While not hidden antigens, filarial excretory-secretory products have been highly studied both as vaccine candidates and for their ability to modulate host immune responses. Characterizations of ES products of *B. malayi* have previously been performed (20), yet it has never been clear which organ within the worm produces each ES product. ES products can be derived from the worm's surface coat or be secreted or excreted from various orifices in including the secretory pore, mouth, anus, and vulva (85; 108). The structures within the worm that are associated with the production of these ES products are the pharyngeal gland, secretory glands within the lateral cords, and possibly the intestinal tract. Although this study does not determine the exact location of production of where individual ES products are made, it does help to narrow down the location of production of some specific ES products to an anatomic fraction. Of the 227

adult female ES products, we found that 49 were enriched within the uterine tubes, 8 in the gut, and 11 in the body wall.

In conclusion, this manuscript details the proteins found within the major anatomic fractions of *B. malayi,* including the digestive tract, body wall, and reproductive tract. 75 named proteins were identified that were enriched within or specific to the gut of *B. malayi* and were predicted to be secreted or have trans-membrane domains. This group included many transporters and enzymes, including 5 proteolytic enzymes that may be targeted for future research.



Supplementary Figure 1: Number of peptides for each identified cuticle collagen within each of the anatomic fractions.



Supplementary Figure 2: A) Cuticle collagens enriched within the uterine tubes of the adult female were analyzed against previous data for their presence within the adult female (AF), Microfilariae (MF), and Uterine Tube derived microfilariae, (UTMF). B) Number of peptides for each of the trehalase family proteins within the anatomic fractions.

Supplementary Table 1: Most abundant proteins in the gut of adult female *B. malayi*

Protein	Gut	Body wall	UT	Quant. Value	Role
TIGR_14973_m02610 oxidoreductase, short chain dehydrogenase/reductase family protein 938 bp	88	90	18	128.22	Lipid Metabolism
TIGR_14977_m04983 heat shock 70 kDa protein, putative 679 bp	56	51	80	112.09	Chaperone
TIGR_15463_m00018 cytoplasmic intermediate filament protein, putative 321 bp	34	55	32	106.37	Cytoskeletal
TIGR_14992_m11078 heat shock protein 90, putative 699 bp	54	36	76	105.96	Chaperone
TIGR_14975_m04318 Glyceraldehyde 3-phosphate dehydrogenase, putative 339 bp	31	41	33	103.92	Glycolysis
TIGR_14258_m00140 actin, putative 376 bp	13	7	12	96.776	Cytoskeletal
TIGR_14920_m00400 Spectrin alpha chain, putative 2423 bp	148	116	158	94.121	Cytoskeletal
TIGR_14977_m04971 cytoplasmic intermediate filament protein, putative 287 bp	22	29	17	89.834	Cytoskeletal
TIGR_14594_m00163 actin 1, putative 376 bp	41	83	35	87.384	Cytoskeletal
TIGR_14594_m00167 actin 2, putative 376 bp	3	6	3	85.955	Cytoskeletal
TIGR_14965_m00432 actin 2, putative 376 bp	1	2	1	81.259	Cytoskeletal
TIGR_14979_m04552 intermediate filament protein, putative 573 bp	44	57	34	78.4	Cytoskeletal
TIGR_14731_m01012 galectin, putative 280 bp	29	32	33	76.767	Lectin
TIGR_14703_m00079 enolase, putative 436 bp	30	38	32	72.275	Glycolysis
TIGR_14981_m02389 galectin, putative 277 bp	19	19	17	70.846	Lectin
TIGR_13659_m00015 glyceraldehyde 3-phosphate dehydrogenase 209 bp	2	1	2	68.192	Glycolysis
TIGR_14634_m00551 probable spectrin beta chain, putative 4014 bp	138	54	131	59.413	Cytoskeletal
TIGR_14773_m00919 beta-tubulin, identical 448 bp	34	42	50	57.984	Cytoskeletal
TIGR_14894_m00090 elongation factor 1-alpha (EF-1-alpha), putative 513 bp	25	18	30	55.33	Translation
TIGR_14260_m00125 collagen alpha 2(IV) chain precursor, putative 1761 bp	37	34	46	55.33	Basement Membrane

Supplementary table 2: Most abundant proteins in the body wall of adult female *B. malayi*

	Gut	Body wall	UT	Quant. Value	Role
TIGR_14594_m00163 actin 1, putative 376 bp	41	83	35	8,926.00	Cytoskeletal
TIGR_14972_m07860 myosin heavy chain, putative 1957 bp	86	181	104	4,010.60	Cytoskeletal
TIGR_14979_m04552 intermediate filament protein, putative 573 bp	44	57	34	3,975.40	Cytoskeletal
TIGR_14594_m00167 actin 2, putative 376 bp	3	6	3	3,478.10	Cytoskeletal
TIGR_14992_m10936 Myosin tail family protein 1985 bp	63	168	76	3,372.30	Cytoskeletal
TIGR_14258_m00140 actin, putative 376 bp	13	7	12	3,019.00	Cytoskeletal
TIGR_15463_m00018 cytoplasmic intermediate filament protein, putative 321 bp	34	55	32	2,758.30	Cytoskeletal
TIGR_14977_m04971 cytoplasmic intermediate filament protein, putative 287 bp	22	29	17	2,528.60	Cytoskeletal
TIGR_13004_m00071 Paramyosin, putative 647 bp	31	67	37	2,432.80	Cytoskeletal
TIGR_14975_m04318 Glyceraldehyde 3-phosphate dehydrogenase, putative 339 bp	31	41	33	2,378.30	Glycolysis
TIGR_14965_m00432 actin 2, putative 376 bp	1	2	1	2,262.30	Cytoskeletal
TIGR_14972_m07587 protein unc-22, putative 6781 bp	49	268	29	2,106.60	Muscle regulation
TIGR_14703_m00079 enolase, putative 436 bp	30	38	32	1,853.80	Glycolysis
TIGR_14972_m07771 Disorganized muscle protein 1, putative 321 bp	17	42	20	1,664.50	Cytoskeletal
TIGR_12509_m00023 myosin heavy chain B (MHC B), putative 612 bp	18	47	20	1,664.00	Cytoskeletal
TIGR_14973_m02610 oxidoreductase, short chain dehydrogenase/reductase family protein 938 bp	88	90	18	1,629.30	Lipid metabolism
TIGR_14231_m00144 carbohydrate phosphorylase, putative 838 bp	37	73	27	1,240.70	Glycogen Catabolism
TIGR_14731_m01012 galectin, putative 280 bp	29	32	33	1,139.20	Lectin
TIGR_14176_m00093 fructose-bisphosphate aldolase 1, putative 363 bp	30	43	35	922.44	Glycolysis
TIGR_14047_m00010 myosin heavy chain B (MHC B), putative 599 bp	28	51	29	916.29	Cytoskeletal

Supplementary table 3: Most abundant proteins in the uterine tubes of adult female *B. malayi*

	Gut	Body wall	UT	Quant. Value	Role
TIGR_14977_m04983 heat shock 70 kDa protein, putative 679 bp	56	51	80	117.24	Chaperone
TIGR_14992_m11078 heat shock protein 90, putative 699 bp	54	36	76	104.8	Chaperone
TIGR_12698_m00328 histone H2A, putative 126 bp	8	4	19	100.38	Histone
TIGR_14773_m00919 beta-tubulin, identical 448 bp	34	42	50	81.435	Cytoskeletal
TIGR_12698_m00330 histone H4, putative 103 bp	10	7	17	75.49	Histone
TIGR_14260_m00125 collagen alpha 2(IV) chain precursor, putative 1761 bp	37	34	46	74.384	Collagen
TIGR_14975_m04318 Glyceraldehyde 3-phosphate dehydrogenase, putative 339 bp	31	41	33	69.96	Glycolysis
TIGR_14258_m00140 actin, putative 376 bp	13	7	12	64.706	Cytoskeletal
TIGR_14703_m00079 enolase, putative 436 bp	30	38	32	62.77	Glycolysis
TIGR_14920_m00400 Spectrin alpha chain, putative 2423 bp	148	116	158	61.111	Cytoskeletal
TIGR_14594_m00163 actin 1, putative 376 bp	41	83	35	56.825	Cytoskeletal
TIGR_14594_m00167 actin 2, putative 376 bp	3	6	3	54.336	Cytoskeletal
TIGR_14500_m00158 histone H2A, putative 126 bp	1	0	2	53.645	Histone
TIGR_14972_m07435 tubulin alpha chain - mouse, putative 448 bp	38	42	53	53.507	Cytoskeletal
TIGR_14500_m00157 Probable histone H2B 3, putative 211 bp	8	4	16	52.954	Histone
TIGR_14965_m00432 actin 2, putative 376 bp	1	2	1	50.189	Cytoskeletal
TIGR_14972_m07646 Endoplasmin precursor, putative 789 bp	52	38	58	49.221	Protein modification
TIGR_14894_m00090 elongation factor 1-alpha (EF-1-alpha), putative 513 bp	25	18	30	48.944	Translation
TIGR_13659_m00015 glyceraldehyde 3-phosphate dehydrogenase 209 bp	2	1	2	42.031	Glycolysis
TIGR_14731_m01012 galectin, putative 280 bp	29	32	33	40.787	Lectin

Supplementary table 4: Gut enriched proteins predicted to be secreted or have transmembrane domains.

Protein	# Peptides			Transmembrane Domain
Carbohydrate Metabolism	Gut	Body Wall	UT	
hexokinase, putative	6	0	3	1
epimerase/reductase-related	2	0	0	
UDP-N-acetylglucosamine-dolichyl-phosphate N-acetylglucosaminephosphotransferase, putative	3	0	1	10
Cell Adhesion				
Immunoglobulin I-set domain containing	3	1	0	1
Immunoglobulin I-set domain containing	4	0	2	1
EGF-like domain containing protein	3	0	0	1
Cell structure				
MGC26979 protein-related	2	1	0	4
Cell Membrane Structure				
SacI homology domain containing	2	1	0	
Autophagy protein Apg9 containing	4	1	0	5
Cell Signaling				
Tyrosine-protein kinase abl-1.-related	3	0	1	1
Protein kinase domain containing	2	0	1	
Protein kinase domain containing	5	1	1	1
Ser/Thr protein phosphatase family	4	1	0	1
Innexin family protein	3	0	0	3
Ground-like domain containing protein	2	0	0	
Ground-like domain containing protein	2	0	1	
SKI/SNO/DAC family protein	3	0	1	
phosphatidate cytidylyltransferase-related	2	0	1	3
Putative phosphatidate cytidylyltransfer0ase, putative	5	2	0	8
Latrophilin receptor protein 2,	2	1	0	7
Patched family protein	4	1	1	9
protein C24B5.3 , putative	6	3	0	12
Ly-6-related protein HOT-2-related	2	0	0	2
sulfakinin receptor protein, putative	2	0	0	5
Chaperones/HSP				
DnaJ domain containing protein	6	0	1	1
DnaJ protein, putative	2	0	1	

DNA/RNA Binding				
Zinc finger DHHC domain	2	1	0	4
Zinc finger, C2H2 type	2	1	0	
Glycine-rich RNA-binding protein.-related	2	1	0	
Drug metabolism				
Cytochrome P450 family protein	2	0	0	
Fatty Acid Metabolism				
Fatty acid desaturase family	4	1	1	4
Glycosylation/glucuronidation				
glycosyl transferase, group 2	2	1	0	1
Fukutin.-related	2	0	1	1
UDP-glucoronosyl and UDP-glucosyl transferase	19	2	0	1
Hydrolases				
Histidine acid phosphatase family	2	0	0	
Histidine acid phosphatase family	12	3	1	
Sulfatase family protein	2	0	1	
Immunological				
Complement component C6 precursor.-related	2	0	1	1
Lectins				
Lectin C-type domain containing	2	0	1	1
Miscellaneous				
Acyltransferase family protein	5	1	1	12
cDNA sequence BC017158-related	3	0	1	4
CG3054-PA-related	2	0	1	2
D4Ertd196e protein, putative	3	0	1	2
DB module family protein	2	0	0	
excretory/secretory protein Juv-p120 precursor-related	5	0	2	
MGC69076 protein-related	7	1	1	
Protein C20orf142 homolog precursor.-related	2	0	0	6
Peroxisome assembly				
Peroxin-3 family protein	4	2	0	
Protease inhibitor				
serpin, putative	2	1	0	1
Proteases				
metalloprotease, putative	2	1	0	
Papain family cysteine protease	8	0	0	1
aspartic protease BmAsp-1, identical	8	3	0	

Peptidase family M1 containing	2	0	0	1
Rhomboid family protein	2	0	1	7
Sterol homeostasis				
Oxysterol-binding protein	4	1	1	1
Structural				
Nematode cuticle collagen N-terminal	2	0	1	1
Transporters				
ABC transporter N-terminus family	2	0	0	3
ABC transporter transmembrane region	2	0	0	3
Cation transporter family protein	2	0	0	4
Ctr copper transporter family	3	1	0	3
E1-E2 ATPase family protein	2	1	0	5
ZIP Zinc transporter family	3	0	0	6
AMOP domain containing protein	46	2	14	1
NRAMP-like transporter K11G12.4, putative	7	0	2	5
Twik family of potassium	5	0	1	6
TWiK family of potassium	3	1	0	6
TWiK family of potassium	4	0	2	6
Major Facilitator Superfamily protein	8	0	2	9
Major Facilitator Superfamily protein	4	0	2	10
cation efflux family protein	2	1	0	5
Transmembrane amino acid transporter	2	1	0	9
Mitochondrial carrier C16C10.1, putative	2	0	0	4
Probable mitochondrial import receptor	2	0	0	1
RE11181p-related	2	1	0	5
zgc:92765, putative	4	0	1	10

Supplementary table 5: Gut enriched ES products

Accession number	Protein	Gut	BodyWall	Uterine Tube
14387.m00341	Papain family cysteine protease	8	0	0
12579.m00069	hypothetical protein	4	2	1
14972.m07360	Patched family protein	4	1	2
14961.m05227	Hypothetical 65.5 kDa Trp-Asp	3	1	1
13350.m00129	conserved hypothetical protein	3	0	1
14518.m00086	hypothetical protein	2	0	1
14972.m07024	fumarylacetoacetate hydrolase domain containing	2	0	1
14979.m04395	Zinc finger protein, putative	2	0	1

Supplementary table 6: Body wall enriched ES products

Accession Number	Protein	Gut	Body Wall	Uterine Tube
14992.m10936	Myosin tail family protein	63	168	76
14972.m07587	protein unc-22, putative	49	268	29
13877.m00097	Immunoglobulin I-set domain containing	15	148	23
14992.m10878	Aminopeptidase W07G4.4 in chromosome	14	24	9
14972.m07803	Cuticular glutathione peroxidase precursor,	12	28	10
14979.m04538	Ryanodine Receptor TM 4-6	9	70	17
13939.m00062	Nematode cuticle collagen N-terminal	6	15	4
14950.m01803	HAD-superfamily hydrolase, subfamily IA,	4	5	0
14538.m00484	Hypothetical protein	1	2	0
14975.m04339	hypothetical protein	0	5	3
13644.m00284	Peptide methionine sulfoxide reductase	0	3	0

Supplementary table 7: Uterine tube enriched ES products

Accession number	Protein	Gut	Body Wall	Uterine Tube
14646.m00062	Phosphatidylinositol 3- and 4-kinase	11	5	33
12698.m00324	Potential global transcription activator	9	2	29
14253.m00150	BTB/POZ domain containing protein	10	4	23
13311.m00340	KH domain containing protein	10	8	21
15451.m00017	FKBP-type peptidyl-prolyl cis-trans isomerase-59,	14	5	21
13464.m00243	Histone deacetylase 1, putative	8	2	18
13501.m00079	U2 auxiliary factor 65	8	2	18
14972.m07815	Lectin C-type domain containing	8	3	16
13987.m00026	hypothetical protein	4	5	14
14271.m00288	hypothetical protein	2	2	13
12849.m00037	Kunitz/Bovine pancreatic trypsin inhibitor	4	1	13
14518.m00083	Biotin/lipoate A/B protein ligase	4	2	13
14980.m02744	Snf5 homolog R07E5.3 -	2	5	13
13825.m00092	G-patch domain containing protein	2	1	12
14990.m08084	tRNA modification GTPase TrmE	3	3	10
14250.m00296	Aspartyl aminopeptidase, putative	1	2	9
14977.m04858	hypothetical protein	4	1	9
14333.m00012	excretory/secretory protein Juv-p120 precursor-related	2	0	8
14972.m07925	conserved hypothetical protein	3	4	8
14519.m00041	RhoGAP domain containing protein	2	5	8
14981.m02423	hypothetical protein	1	2	7
14916.m00475	von Willebrand factor type	1	2	6
14980.m02817	26S proteasome regulatory chain	1	0	5
14972.m07789	Phosphatidylinositol 3- and 4-kinase	0	1	5
14973.m02692	Gex interacting protein protein	0	1	5
14972.m07642	E1-like protein-activating enzyme Gsa7p/Apg7p	0	4	5
14771.m00162	hypothetical protein	0	0	4
14250.m00295	hypothetical protein	1	0	4
14950.m01851	conserved hypothetical protein	1	1	4
13685.m00019	Zinc finger, C2H2 type	1	1	4

14942.m00199	60S ribosomal protein L34,	1	1	4
12737.m00156	Galactosyltransferase family protein	0	2	4
14990.m07725	Alpha-catulin, putative	0	2	4
14943.m00197	NOL1/NOP2/sun family putative RNA	2	1	4
14077.m00079	Helicase conserved C-terminal domain	1	2	4
14364.m00217	hypothetical protein	0	0	3
12491.m00042	RhoGEF domain containing protein	1	0	3
14553.m00039	hypothetical protein, conserved	1	0	3
14974.m00795	hypothetical protein	1	0	3
14351.m00110	Elongation factor Tu GTP	1	1	3
14971.m02786	Golgi autoantigen, golgin subfamily	1	1	3
13138.m00177	hypothetical protein, conserved	0	0	2
14278.m00072	conserved hypothetical protein	0	0	2
14765.m00799	Formin Homology 2 Domain	0	0	2
14975.m04421	Eye-specific diacylglycerol kinase, putative	0	0	2
14947.m01148	Trypsin Inhibitor like cysteine	1	0	2
14990.m07720	Ras family protein	1	0	2
15200.m00009	RNA recognition motif.	1	0	2
14284.m00378	hypothetical protein	0	1	2

Supplementary table 8:Identification of proteins shown previously to be protective in permissive models of *Brugia malayi*

% Protection	Antigen	Accession Number	Current Protein Name	GUT	BODY WALL	UTERINE TUBE
64%	rMyosin	14972.m07860	myosin heavy chain, putative	86	181	104
30%	Transglutaminase	14937.m00487	transglutaminase, putative	25	29	36
43%	Thioredoxin Peroxidase	14972.m07237	Thioredoxin peroxidase 1, putative	3	4	5
76%	BmALTI	14933.m00224	unknown, identical			
69%	BmALTII	15013.m00154	conserved hypothetical protein			
82.75	Glutathione-S-Transferase	14961.m05283	glutathione transferase, putative	10	13	10
39%	Superoxide Dismutase	14990.m07729	Superoxide dismutase , mitochondrial			
	Vespid Allergen Homolog	14046.m00191	venom allergen antigen-like protein			
43%	Paramyosin	13004.m00071	Paramyosin, putative	31	67	37
64%	BmSL3	14058.m00567	60S ribosomal protein L4,	13	16	18

Chapter 5: Discussion

MAJOR FINDINGS

The specific aims of these studies were: first, determine the safety and efficacy of a vaccine comprised of the soluble fraction of *L. sigmodontis* intestinal tract, second, to determine differences in and evaluate importance of humoral and cellular immune responses in a protective compared to a non-protective vaccine, third, to determine antigens within the digestive tract of *B. malayi* that could be possible targets for future vaccine research. These specific aims were accomplished within this study. For the first specific aim we found that despite the soluble fraction of the digestive tract of *L. sigmodontis* displaying some hidden qualities, allergies were still a concern with this approach. Vaccination with the soluble intestinal antigen provided no statistical protection against either challenge infection or patency. For the second specific aim, we found that humoral responses were not necessary for protection, and that protected mice, vaccinated with irradiated L3, produced both type I and type II cytokines. Despite various vaccine protocols using soluble adult antigen, we were unable to replicate this mixed cytokine response or protective immune responses. Finally, for the third specific aim, we found 5 proteolytic enzymes, a serpin, some histidine acid phosphatases, a sulfatase, and many transporters and enzymes specific to the *B. malayi* intestinal tract that may be candidates for future vaccine work.

THE SOLUBLE FRACTION OF FILARIAL INTESTINAL ANTIGENS EXHIBITS HIDDEN QUALITIES, BUT MAY STILL POSE A RISK FOR ALLERGIC REACTION IN EXPOSED INDIVIDUALS.

The antigens obtained from the intestines of nematodes have been previously shown to be immunologicaly hidden. This is the case to some extent in dogs infected with *D. immitis*, and to a great extent in sheep infected with *H. contortus*. Consistent

with this, we found that both IgG antibody titers and splenocyte cytokine responses to intestinal antigens were lower than the IgG antibody titers and splenocyte cytokine responses to the soluble fraction of adult worms.

Despite this, the intestinal antigens were not completely hidden. IgG antibody titers to the intestinal antigens of *L. sigmodontis* were still present in infected mice, and splenocytes from infected mice still produced cytokines in response to these antigens. While overall these findings suggest that perhaps some of the antigens present within the intestines of *L. sigmodontis* may be hidden from infected mice, there are at least some antigens present within the soluble fraction of the intestines of *L. sigmodontis* that are recognized by host adaptive immunity.

These observations highly consistent with the data obtained from the proteomic analysis of the gut, body wall, and uterine tubes of *B. malayi*. The majority of proteins that were found within the digestive tract of *B. malayi* were also present in the other two anatomic fractions in this parasite. Furthermore, even proteins, such as the Papain family cysteine protease, that were highly enriched within the gut of the worm have previously been found within the ES products of adult females.

This lack of perfect sequestration from the adaptive arm of the immune system does not rule out the possibility of good vaccine candidates among the intestinal antigens of filarial worms. Instead, there is clear evidence from many different helminths that intestinal antigens can be protective. Success using this vaccine approach in filariasis, however, is going to require a strong understanding of filarial biology and a targeted approach.

This is highlighted by the similar levels of IgE antibodies to intestinal antigens as to the soluble fraction of whole worms. IgE antibodies are one of the major obstacles to a safe filarial vaccine (44). Furthermore, the basophil activation test showed that 8 week infected mice exhibited similar levels of basophil activation in response to GutAg as to LsAg. The reason for the similarity of allergy associated immune responses in GutAg vs. LsAg when IgG antibodies were so much lower to the soluble fraction of the gut is not entirely clear.

However, the presence of proteolytic enzymes within the intestine of the worm that eventually become ES products may be the cause. The adaptive immune response has a predilection for developing allergic type reactions to proteolytic enzymes. Furthermore, of the 5 proteolytic enzymes that are highly enriched within the digestive tract of *B. malayi*, 3 of them have a homology to human proteins that falls between 30-40%. This specific range of homology to host proteins is suggested to be more likely to induce allergic type reactions (128). Therefore, the structure and function of some of the key enzymes within the intestinal tract of filarial worms may be the cause of the relatively high allergy associated immune responses.

VACCINATION WITH THE SOLUBLE FRACTION OF INTESTINAL ANTIGENS IS NOT PROTECTIVE AGAINST CHALLENGE INFECTION OR PATENCY.

Previously, it had been shown in a mouse model of *D. immitis* that mice vaccinated with the soluble fraction of intestinal worm antigen exhibited an increased ability to kill infective larvae. This protective effect was not present, or not as high, when more insoluble fractions of the intestine were used to vaccinate mice. This was the only previous model of filariasis that had examined the effects of vaccination with any intestinal antigens from a filarial worm. We attempted to follow up on this study using

the permissive *L. sigmodontis*/BALB/c model of filariasis. However, in our study, mice that were vaccinated three times with 10μg GutAg using CpG/Alum as an adjuvant showed no decrease in either adult worm burdens or microfilaremia 8 weeks post challenge infection.

In order to examine the reason for the lack of protection in this model, it is important to understand the past successes of intestinal antigen vaccines and their mechanism of action. The *D. immitis* study was unique compared to other helminth intestinal antigen studies in that it found protection with the soluble fraction of the intestine. In many of the other studies, protection has been elicited by vaccination with the membrane bound glycoproteins. It is thought that these glycoproteins are present within the brush border of the parasite's intestinal tract, and are directly responsible for degrading hemoglobin after hematophagy. Indeed, many of the parasitic helminths that have shown to be affected by vaccination with helminth derived intestinal antigens rely heavily on hematophagy. Furthermore, most helminths that rely heavily on hematophagy have very large microvilli and a very well developed intestine. For instance, the intestine of *H. contortus* makes up a very large part of the worm, and can easily be seen on any worm images.

In contrast to this, *L. sigmodontis* only engages in hematophagy during a transient period around the molt to adulthood. The intestines of the worm do not make up much of the worm, and only consist of a simple epithelium on top of a basement membrane. It is possible that the failure of this vaccine to protect against *L. sigmodontis* may be due to a decreased need of this parasite to heavily digest its nutrients. It is, however, also likely that instead of the soluble fraction of the intestines, selectively vaccinating with the

membrane bound glycoproteins, as was done with *H. contortus* would provide better results. Of note, it has been shown that parasitic nematodes that do not engage in hematophagy ingest host antibodies directed against intestinal antigens(103).

VACCINATION WITH THE SOLUBLE FRACTION OF ADULT WORMS DOES NOT RESULT IN PROTECTIVE IMMUNE RESPONSES.

In some non-permissive models of filariasis, such as mice infected with *B. malayi* there have been studies showing that vaccination with almost any worm antigen results in a protective immune response against the worm. Although the *L. sigmodontis/*BALB/c model of filariasis is permissive, the adult worms are cleared out much sooner than in the natural host, and there remained the possibility that this model would not be a robust one for vaccine screening. Vaccination with 10 μg of LsAg using either alum or CpG/alum as an adjuvant did not result in significant protection against challenge infection. Similarly, vaccination with 50μg of LsAg adsorbed to alum was not protective. The major significance of this is that it suggests that protection in this model, like that in other permissive models, is not easily induced. For this reason, this model is likely an effective one for screening vaccine candidates.

Besides providing some more information on the model, the lack of protection by vaccinating with LsAg provides the opportunity to compare non-protective parasite directed immune responses to protective ones. In previous studies, it has been shown that IL-5 is necessary for protection after iL3 vaccination. In a follow up study with IL-5 transgenic mice, it was shown that overproduction of IL-5 by mice results in protection against *L. sigmodontis.* While this provides some information into methods of parasite killing, it does not necessarily mean that the level of IL-5 induced by vaccination with irradiated larvae is in itself sufficient to induce a protective immune response. The initial

vaccine experiment where mice vaccinated with LsAg using CpG/alum as an adjuvant exhibited no protection suggested 0that perhaps Type II cytokines levels in iL3 vaccinated mice were responsible for the protective effect.

However, a vaccine regimen with LsAg adsorbed to high dose alum induced both IL-4 and IL-5 responses to worm antigens, but was not protective against challenge infection. This suggests that more than just IL-5 responses are responsible for the protective response.

LIMITATIONS OF THESE STUDIES

It must be stated that the tools used in these studies were the tools available and are imperfect. In the cytokine studies that were carried out, there were three specific factors that could induce error. First, the protective iL3 vaccination protocol required a subcutaneous injection, while we utilized IP vaccination for the LS vaccine. Second, the cells used for stimulation were pulled from the spleen and may not be representative of the immune response that occurs within the pleural cavity. Third, the soluble fraction of adult worms was used to stimulate these cells. It is possible that an immune response to insoluble parasite antigens may induce a different response, and it is possible that antigens within a different stage of the life cycle may induce a different response. Indeed, the time course experiments with the irradiated larval vaccine suggest that parasite clearance occurs mostly within the L4 stage. Further work on this stage is feasible, as worms in the L4 stage are large enough to obtain sufficient quantities of antigen for immunological studies.

VACCINATION WITH IRRADIATED LARVAE MAY INDUCE DIFFERENT MECHANISMS OF PROTECTION DEPENDING ON SOURCE OF LARVAE

One of the more surprising findings in this study was that the irradiated larval vaccine that we used likely protected against challenge infection through a different

mechanism than has been seen in other labs. The two major differences were that the majority of worm clearance occurred much later in our studies, and that antibodies were not necessary for protection. In other laboratories, it was shown that vaccinated mice harbored much fewer worms as early as 3 days post infection, and that the levels of protection did not change after that period. Furthermore, it had been shown that antibody deficiency completely abrogated the protective effect of iL3 vaccination. After thorough review of the protocols the only difference that had been found was the source of infective larvae. In our lab, we utilize larvae that have been obtained through a pleural lavage of infected jirds 4 days post infection. For infection, this protocol is ideal as it results in much higher levels of surviving larvae and a higher percentage of mice that become microfilaremic. However, vaccination with mammalian derived iL3s also results in a completely different mechanism of protection after vaccination tan observed with vector derived iL3s.

The possibilities of this are immense. Recent vaccine work in filariasis has been focused on vaccines that target different parts of the worm life cycle in a hope to finally reach sterilizing immunity. Since vaccination with mammalian derived iL3s shows a difference in the method of protection, it provides another lifecycle stage that can be targeted. However, the problem with this finding is that it is now possible that neither irradiated larval vaccine is as well understood as was previously thought. For example, the mite derived irradiated larval vaccine has been shown to be protective for a period of at least 6 months post vaccination, but this information is not known for the vaccine protocol used in our lab. Similarly, with the jird derived iL3 vaccine, it has been shown that repeated parasite exposure post vaccination did not reduce the protective effect of the

vaccine. With the information that these vaccine protocols are different, it is now not known if the mite derived irradiated larval vaccine will also perform adequately after repeated challenge infection.

ANTIBODIES ARE NOT NECESSARY FOR NATURAL PROTECTION AGAINST *L. SIGMODONTIS* IN BALB/C MICE

BALB/c mice are considered permissive to infection with *L. sigmodontis* because infective larvae are capable of infecting these mice, reaching the adult stage and producing MF. However, mice still clear these infections. In our model, about 50% of infective larvae reach the adult stage, and the percent survival of parasites remains steady until approximately 10 weeks PI. At this time, there is a dramatic worm decrease in worm survival, to approximately 12% of the original inoculum. Parasite burdens then decrease slowly until the mouse has cleared the infection, typically by 20 weeks PI. The clearance of adult worms in this model has been shown to be dependent on IL-5 and IFNγ (127; 153). Using μMT mice, previous studies had suggested that antibodies were not necessary for this clearance to occur. However, the finding that μMT mice still produced IgE antibodies after infection with helminths (114) made it unclear whether or not antibodies truly played no role in the previous studies. Using J_H mice, which do not produce antibodies during helminth infection (114), we showed that antibodies are not necessary for the decline in worm survival that occurs 10 weeks PI.

ANTIBODIES CAN BE TARGETED TO FIND A PROTECTIVE IMMUNE RESPONSE.

If antibodies can be protective against filariasis, this would by far be the easiest immune response to try to understand and then replicate. Many methods exist of determining which antigens are targeted by host antibodies, and some of these studies are high throughput. Although one of the major findings in this study was that antibodies were not necessary for protection conferred by the jird derived irradiated larval vaccine,

two points suggested that antibodies could be sufficient to clear filarial worms. First, larvae pre-incubated in plasma from iL3 vaccinated mice showed a decreased ability to survive after being injected into naive mice. Second, although iL3 vaccinated J_H mice were still protected against challenge infection, they exhibited lower levels of protection 4 weeks post challenge. Combined, this suggests that antibodies play some role in protection against challenge infection. Additionally, prior studies have shown that the mite derived iL3 vaccine was completely dependent on antibodies for protective efficacy. For these reasons, future research into the antibody profile of protected iL3 vaccinated mice and sensitized LsAg vaccinated mice may be fruitful for future vaccine research.

Another, potentially complementary approach is to specifically target antigens within the worm intestine. This approach has a very strong precedent. In filariasis though, it was previously unclear which proteins were located only within the digestive tract of *B. malayi*. We found 75 proteins enriched within the digestive tract of the worm that were likely to be secreted or trans-membrane. Of these, the 5 proteolytic enzymes are possible future vaccine candidates.

IT IS POSSIBLE THAT WE HAVE THE TOOLS TO PRODUCE A VACCINE AGAINST FILARIASIS

The combination of a strong review of the literature and data obtained through experiments strongly suggests that multiple mechanisms can be employed in vaccine development against filariasis. At least 10 different individual antigens have shown some protection against *B. malayi* in a permissive model. Furthermore, the combination of different individual antigen vaccines to achieve higher levels of protection than either vaccine alone suggests that perhaps the right combination would allow for sterilizing immunity. Even if sterilizing immunity is impossible, vaccines that decrease

pathological sequelae or prevent transmission may prove useful against these terrible diseases.

However, the production of a vaccine against human filariasis is fraught with difficulty. Vaccine production is expensive, and any vaccine candidate would have to go through extensive testing to ensure no exacerbation of pathology. Testing the efficacy of any vaccine against filariasis may be currently infeasible because of the extensive eradication campaigns. Furthermore, it is debatable whether vaccine trials would be an appropriate use of funds while ongoing mass drug administration may prove fruitful in eradicating these diseases.

For these reasons, it would be more advantageous to show proof of concept of vaccination in a disease model where a vaccine could be beneficial in a short amount of time. Surprisingly, such a model exists in filariasis, and it seems likely that a vaccine could be utilized in this model. Infection of dogs with the filarial worm *Dirofilaria immitis* results in severe disease and is a common concern of pet owners. Current control measures rely on prophylaxis with anthelmintics; however, there have been cases where prophylaxis has failed. This is possibly the manifestation of a developing anthelmintic resistance in some strains of *D. immitis*.

The possibility of being able to induce a protective immune response in dogs against *D. immitis* is high because vaccination with irradiated larvae and chemically abbreviated infections have both shown very high levels of protection (56; 159; 162). Finally, it is not necessary for a vaccine to induce sterilizing immunity in this model to provide some benefit. Disease status with this infection is directly related to worm burden. Therefore, decreasing worm numbers would have a beneficial effect against

disease status. The development of a vaccine against *D. immitis* is likely possible, could be developed quickly, would be beneficial in its own right, and would provide great insight into future vaccine work in humans.

PROPOSED FUTURE WORK

In order to find new vaccine targets, we propose that a high throughput method of determining proteins recognized by protected (mite derived iL3 vaccinated) mice be compared to non-protected (LsAg) vaccinated mice. We suggest mite derived iL3s because this vaccination protocol is dependent on antibodies for protection. Worm antigens could be obtained from the L3, L4, and adult stages and run through a column containing stationary antibodies from LsAg vaccinated mice or mite derived iL3 vaccinated mice. The bound antigens could be eluted off and run through RPLC/MS-MS to determine antigens that were bound by host antibodies. A comparison of antigens bound by protected and non-protected mice may provide some insight into future vaccine candidates.

Furthermore, we propose that transmembrane individual intestinal antigens, especially the intestinal specific proteolytic enzymes, could be targeted by antibodies. We propose initially screening with DNA vaccines containing sequences from these proteins of interest. Any that show particular promise could be further evaluated by vaccination with recombinantly expressed proteins.

Lastly, we propose that the development of a vaccine may be accomplished by taking known protective antigens and empirically using combinations of these antigens while varying adjuvants in a dog model of *D. immitis.* Although this is not a perfect model of human disease, a vaccine against *D. immitis* would be beneficial in animal companion medicine and likely decrease the number of infections in humans by

preventing transmission in dogs. Furthermore, it is possible that a combination of antigens with high homology to proteins in filarial pathogens in humans would also provide protection for people.

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