

Evaluating Intestinal Proteins of Adult *Brugia malayi* Worms
as Drug and Vaccine Targets

by

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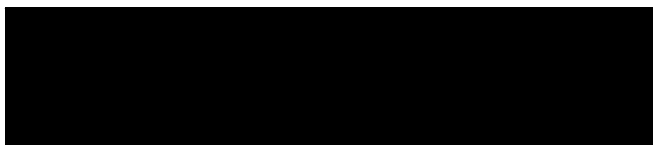
To Bellamie,

Thank you for all your love and encouragement.

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ABSTRACT

Evaluating Intestinal Proteins of Adult *Brugia malayi* Worms as Drug and Vaccine Targets:

Alexander F Flynn, Doctor of Philosophy, 2018

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Lymphatic filariasis (LF), a debilitating disease caused by the tissue-invasive nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, affects over 70 million people worldwide. Global eradication efforts have significantly reduced worldwide prevalence, but complete elimination has been hampered by limitations of current antifilarial drugs and the lack of a vaccine.

The goal of this study was to evaluate *Brugia malayi* intestinal proteins as potential drug target and vaccine candidates. We first evaluated whether *Bma-iUGT* is essential for adult filarial worms by inhibiting its expression with siRNA for 24 hrs. This resulted in a 75% knockdown of *Bma-iugt* mRNA for 6 days and almost complete suppression of detectable *Bma-iUGT* by Western blot. Reduction in *Bma-iUGT* expression resulted in decreased worm motility for 6 days, 70% reduction in microfilaria (Mf) release from adult worms, and significant reduction in adult worm metabolism as detected by MTT assay. We then tested UGT inhibitors,

sulfinpyrazone and probenecid, for *in vitro* activity against *B. malayi*. There was a marked macrofilaricidal effect with the lowest concentrations resulting in reduced worm motility being 200 μ M for sulfinpyrazone and 250 μ M for probenecid. Almost no microfilaricidal effect was observed *in vitro* at 250 μ M for probenecid. Because prior sensitization to a filarial antigen would be a contraindication for its use as a vaccine candidate, we tested plasma from infected and endemic normal populations for *Bma*-iUGT-specific IgE using a luciferase immunoprecipitation assay. All samples (n=30) tested negative.

When we evaluated 8 other intestinal proteins in adult *B. malayi* worms, we found another promising candidate. We observed a 70.42% knockdown of *Bma-lad-2* mRNA 1-day post-siRNA incubation. This inhibition of *Bma*-LAD-2 expression resulted in a robust decrease in worm motility and Mf release as well as an 83.25% reduction in MTT metabolism. By transmission electron microscopy, we visualized the loss of microvilli and unraveling of mitochondrial cristae in worms treated with *Bma*-LAD-2 siRNA. We did not detect any pre-existing *Bma*-LAD-2-specific IgE in the serum of filarial patients.

The results of this study demonstrate that *Bma*-iUGT and *Bma*-LAD-2 are essential proteins for adult worm survival. Lack of prior IgE sensitization in infected and endemic populations suggest it may be a feasible vaccine candidate. The finding that UGT inhibitors sulfinpyrazone and probenecid have *in vitro* effects against adult *B. malayi* worms suggests that these medications have promise as potential macrofilaricides in humans.

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Chapter 1: Introduction

LYMPHATIC FILARIASIS

Over 70 million people worldwide are afflicted with lymphatic filariasis (LF) in tropical and-sub-tropical regions [1]. The clinical manifestations of the disease are varied but can include chronic lymphedema, elephantiasis, and hydrocele [2]. Those afflicted with the disease suffer disability, social ostracization, and economic hardship. The parasitic nematode, *Wuchereria bancrofti*, is the causative agent for 90% of the cases, while *Brugia malayi* and *Brugia timori* are responsible for the remainder [3].

Filaria life cycle

The filaria life cycle consists of 5 stages with humans serving as the definitive host and mosquitos as the vector (Fig 1). The infective stage for humans is the L3 stage during which L3 larvae are delivered by mosquitos during a feeding. The L3 stage larvae quickly migrate to the local lymphatics where they molt into the L4 stage [4]. These immature filariae then move to the central lymphatics where they again molt and develop into adults by 6-9 months post-infection [4]. The adult filariae then release L1 stage larvae or microfilariae (Mf) into the blood. During a subsequent blood meal, mosquitos ingest the Mf, which then penetrate the mosquito's midgut and gain access to the hemolymph [5]. The Mf quickly migrate to the thoracic musculature and penetrate the muscles of the wings [6, 7]. There the Mf undergo 2 molts to become L3 larvae

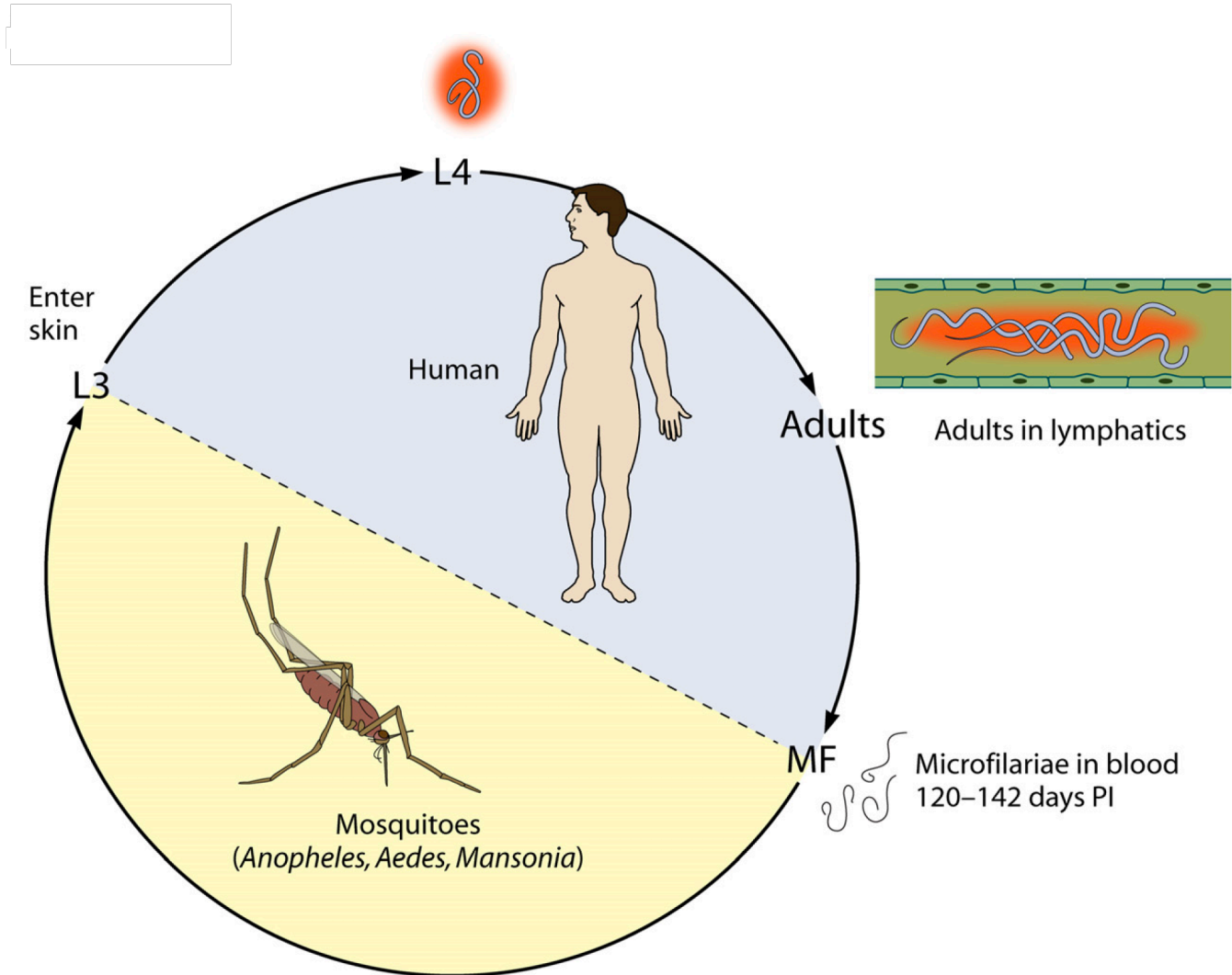


Figure 1. The lifecycle of *Brugia malayi*

that then migrate to the mosquito head and proboscis around 8 days post-exposure [8].

Causative agents of lymphatic filariasis

Wuchereria Bancrofti

W. bancrofti is prevalent in tropical Africa, South East Asia, Papua New Guinea, and the Philippines [9]. These filariae are mainly transmitted by the *Anopheles* mosquito. The Mf display nocturnal periodicity in which the Mf are present in the blood only at night [10]. Compared to those found in other LF species, *W. bancrofti* Mf are typically larger with the body length ranging from 309.2 μm to 346.8 μm . These adult filaria have more protrusive caudal papillae, sensory rays located at the tail, compared to *B. malayi* [11].

Brugia malayi

B. malayi is localized to South and South East Asia where it coexists in some areas with *W. bancrofti* [9]. This filarial species is transmitted by the *Mansonia* mosquitos. In their hosts, *B. malayi* Mf exhibit nocturnal subperiodicity whereby the Mf are present in the blood throughout the day but exhibit a rise in numbers at night [10]. *B. malayi* Mf are generally smaller than those found in other LF species ranging from 205-240 μm in body length. Anatomically, the adult worms, when compared to *W. bancrofti*, have flatter caudal papillae. In addition, these filariae have nuclei that extend to the tip of the tail [11]. Interestingly, cats and non-human primates serve as animal reservoirs for the parasite. This host promiscuity has important implications for the drug and vaccine development.

Filarial research is constrained by the availability of helminth material because of the inability to culture the complete filarial lifecycle *in vitro* [12]. While *W. bancrofti* is the primary cause of LF worldwide [1, 3], the lifecycle cannot be maintained in a laboratory setting [13, 14]. However, the *B. malayi* lifecycle is permissive in experimental hosts such as Mongolian gerbils (jirds), *Mastomys*, monkeys, mice, and ferrets. In our experimental model, jirds are infected using *B. malayi* L3s with patency developing 79 to 116 days post-infection and lasting for at least 26 weeks [15].

Brugia timori

Distribution of *B. timori* is localized to the Lesser Sunda Islands of Indonesia. *Anopheles barbirostris* is the only vector for this parasite [9]. The Mf exhibit nocturnal periodicity in humans. The Mf are also anatomically distinct from other LF species in that the cephalic space length to width ratio is 3:1. Like *B. malayi*, these filariae have nuclei that extend to the end of the tail.

Diagnosis of lymphatic filariasis

It is important to discuss how active infection is best measured and evaluated in humans. The presence of overt pathology does not necessarily prove active infection [16, 17]. In addition, quantifying only microfilaremia levels does not accurately gauge adult worm burden [18]. Mf release into the blood fluctuates and is highly dependent on the time of the blood draw. Furthermore, there are cases involving individuals who are amicrofilaremic but harbor adult worms [19]. Visualization using ultrasonography cannot detect adult worms in deep tissues

[20]. The presence of filarial-specific antibodies does not distinguish between active and previous infection, however, high levels of filarial-specific IgE and IgG4 are common in active infections [21]. A more accurate measure of adult worm burden is detecting adult-specific circulating filarial antigen (CFA) [22-24]. Groups have demonstrated that antigen levels in infected-jird sera correlate well with adult worm burden [25]. In efficacy studies in humans, the antigen tests correctly identified CFA presence in 95% of patients who are microfilaremic and were able to assess infection status in individuals who are amicrofilaremic [24, 26, 27]. Furthermore, CFA is specific to adult worms. Therefore, the absence of infection in humans should be characterized by a lack of microfilaremia and antigenemia.

Subgroups based on clinical manifestation

With lymphatic filariasis (LF) there is a wide range of clinical manifestations that occur in filaria-exposed individuals. Here, we will focus on 4 LF-associated groups: (1) individuals with no signs of infection, (2) individuals with infection that have no chronic pathology, (3) individuals with chronic pathology, and (4) individuals that have tropical pulmonary eosinophilia.

The first group, individuals with no signs of infection, are referred to as *endemic normals* and are considered to be resistant to infection. Studies suggest that the proportion of endemic normals can range from 0% to 90% of a population in endemic areas [28]. However, there is evidence to suggest that there are actually no true endemic normals and that use of sensitive diagnostics can generally detect signs of infection in individuals with extremely low worm burdens

[29]. Alternatively, the absence of infection in some of these individuals could be explained by a lack of exposure to the parasite.

The majority of exposed individuals fall into the second category and show signs of infection but are clinically asymptomatic. Blood analysis of these patients reveals the presence of microfilaria or circulating filarial antigen from adult worms [2, 17]. While there may not be outward signs of disease, these individuals do have subtle signs of lymphatic abnormalities such as vessel dilation, tortuosity, and collateralization as well as increased lymph flow [30-34]. Nevertheless, the lack of overt pathology seen in these individuals is most likely due to a level of tolerance for the worms as the immune response is suppressed with no apparent inflammatory response to the parasites. This is characterized by a downregulation of IL-2 and IFN- γ and shift towards a more regulatory T-cell (IL-10 and TGF- β) response [28, 35]. This shift can be attributed to the well-documented immunomodulatory effect filarial worms can have on the host immune system [36]. In addition, host genetics may play a key factor in whether an infected person remains asymptomatic [2].

Individuals that develop chronic pathology tend to exhibit a strong filarial-specific immune response and few microfilariae [37]. These patients develop hydrocele, lymphodema, elephantiasis, and chyluria (milky white urine) [38-40]. The progression of these clinical symptoms seems to be dependent on the presence of adult filariae. The death of the adult worms triggers an inflammatory response that causes lymphangitis and lymphadenitis in these individuals [17, 28]. The worms are encapsulated in granulomas consisting of macrophages, plasma

cells, eosinophils, neutrophils, and lymphocytes [39]. This is marked by an upregulation of Th1 and Th17 responses and pro-inflammatory cytokines in the host [35]. It is believed that this inflammatory milieu leads to irreversible lymphatic vessel dysfunction and damage, which manifests as progressive lymphedema. In turn, this predisposes the individual to secondary bacterial and fungal skin infections that accelerate the progression of lymphedema and induce the development of elephantiasis [41].

Patients presenting with tropical pulmonary eosinophilia (TPE) have paroxysmal cough and wheezing at night which most likely is due to the nocturnal periodicity of the Mf [2, 17, 28]. These individuals also have a low-grade fever, adenopathy, and elevated levels of eosinophils in the blood. Chest x-rays reveal in lungs of these individuals the presence of bronchovascular markings in some cases miliary lesions [42]. TPE patients typically have elevated total IgE levels and antifilarial titers [43].

Brugia malayi anatomy

B. malayi adult filaria are composed of four major anatomical structures: the body wall, the intestinal tract, the pseudocoelom, and the reproductive tract (Fig 2). The body wall is composed of the surface coat, epicuticle, cuticle, epidermis, and worm musculature [44]. The surface coat is mainly composed of glycoproteins that impart a negative charge. This layer is thought to be derived from secretory glands. It is a dynamic structure as seen by the constant shedding of the surface components by the filaria into the external environment, which is thought to help the worm evade the immune system [45, 46]. The epicuticle is the ridged outer

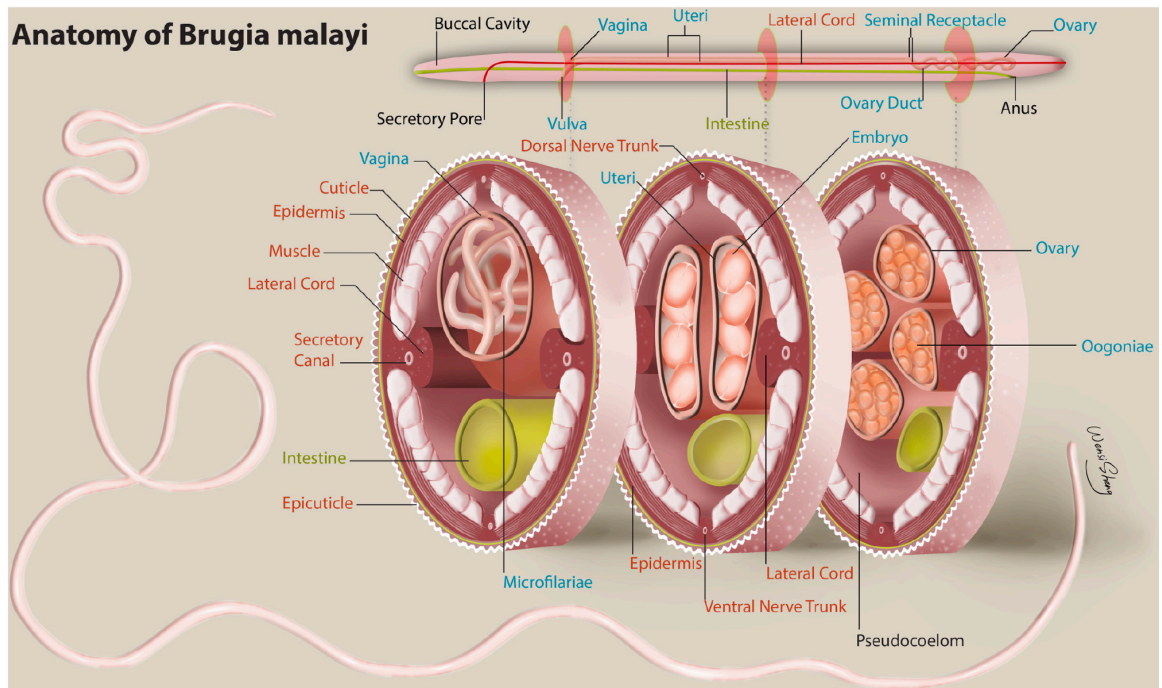


Figure 2. Anatomy of *Brugia malayi* adult worms

most portion of the worm and is described as a trilaminate structure [47]. It is primarily composed of a heterogenous mixture of covalently linked proteins and lipids [44]. Below this structure is cuticle which is mainly composed of cuticulin and collagenous proteins. The cuticle is formed from secretions by the epidermis, which runs just below it. The epidermis is a thin layer of cytoplasm formed from 4 outpouchings containing nuclei that separate the muscle layer into 4 sections [48]. These epidermal pockets are called the lateral, dorsal, and ventral cords. Secretory glands, associated with the lateral chords, have secretory canals that run from the secretory pore near the mouth of the worm to the tail. The dorsal and ventral cords contain nerves that innervate the body wall muscle. Finally, the muscle layer, which is separated by the cords, is composed of up to 9 myocytes for each section [49].

The intestinal tract runs from the mouth of an adult filaria to the anus. It is mainly composed of a single columnar epithelium anchored to a basement membrane [44, 48]. Microvilli are distinctly visible on the surface of the intestine. The pharynx, surrounded by a thin layer of muscle, controls entry of external material into the digestive tract. It is possible that because filariae are under high osmotic pressure, the anus must open and eject intestinal contents before nutrients enter the intestinal tract.

The pseudocoelom is composed of a high osmotic hemolymph, which creates high turgid pressure forming a hydrostatic skeleton for the filaria [44]. Without this, the worms would lose form and eventually be unable to move. In addition, nutrients ingested and absorbed by the filaria are trafficked to cells

through the hemolymph. The hemolymph has high concentration of trehalose and urea in order maintain the high osmolarity.

Filariae are dioecious, but for this project we focused on adult females. Therefore, we will only describe the reproductive tract in that sex. Females have two ovaries at the posterior end which progress into uterine tubes that merge into a single vagina anteriorly [44, 49]. Oocytes, held in meiosis I prophase, progress from the ovaries through the uterine tubes until fertilized by the sperm held in the sperm receptacle [50]. Entry of sperm into the oocyte signals the egg to complete meiosis I and II. The vulva is surrounded by a muscular ovejector that is needed to release the Mf into the external environment [48].

Antifilarials

Diethylcarbamazine (DEC) was first developed by Indian biochemist, Yellapragada Subbarow, in 1947 while he was Director of Research at Lederle [51]. The compound, also known as Hetrazan, is a derivative of piperazine. The WHO recommends administering an annual single dose (6 mg/kg) of DEC to individuals in affected communities [52]. At this dosage, DEC drastically reduces microfilaremia by roughly 90% when measured 6 to 12 months post-treatment, however, the effect on adult worms is limited [53-55]. Despite use as an antifilarial for the past 60 years, the mechanism of action for DEC is not clearly known. It has been suggested that DEC impairs the helminthic neuromuscular system by hyperpolarizing the muscle membranes inducing flaccid paralysis [56]. Also, DEC reportedly causes exsheathment, organelle damage, and cytolysis in *W. bancrofti* microfilariae (Mf) [57]. However, the efficacy of DEC may depend on host factors

since the drug has limited activity *in vitro*. Taylor *et al.* confirmed that DEC targets arachidonic acid metabolism and impairs prostanoid synthesis [58]. Mf constitutively release prostanoids such as PGE₂, PGI₂ and PGD₂ to facilitate passage through the vasculature by preventing adhesion of platelets and endothelial cells [59]. Several groups have also observed that DEC enhances antibody-dependent cell-mediated cytotoxicity by increasing eosinophil adherence [60, 61].

Ivermectin (IVM), also known as Mectizan, was first discovered as collaborative effort between Satoshi Omura of Kitasato Institute in Japan and William C. Cambell of the Merck Institute for Therapeutic Research in the late-1970s [62]. The compound is a derivative of avermectin, which is naturally produced from the bacterium *Streptomyces avermitilis*. At the recommended dosage (150-200 µg/kg), IVM greatly suppresses Mf concentration from 6 to 24 months but has no macrofilaricidal activity [53, 63]. Several studies have demonstrated that IVM causes muscular paralysis by binding to glutamate-chloride channels, which hyperpolarizes neurons [63, 64]. Interestingly, IVM given to microfilariae in culture does not exhibit much microfilaricidal activity [65]. The prevailing thought is that IVM impairs Mf mobility allowing immune effector cells to clear the larvae.

Albendazole, a benzimidazole, was developed in 1975 and has been shown to effectively clear Mf but has negligible macrofilaricidal activity [66]. The compound binds to a colchicine-sensitive site on tubulin and inhibits tubulin polymerization in intestinal cells [67]. This activity hinders cytoskeletal transport and leads to

impaired nutrient uptake and decreased digestion. Together with either DEC or IVM, albendazole can enhance microfilaria clearance by roughly 10% [53, 68].

While not part of the MDA formulary, doxycycline can be used as an alternative for treating LF. The compound is a tetracycline antibiotic and has been shown to have both micro- and macrofilaricidal activity [69, 70]. Doxycycline targets the symbiotic bacteria *Wolbachia* in filaria resulting in sterilization and eventual worm death. However, due to potential disruption of bone growth and development, the drug cannot be given to children under 9 and pregnant or breastfeeding women. This would leave a significant portion of the population untreated.

Global Programme to Eliminate Lymphatic Filariasis

In 1993, the International Task Force for Disease Eradication identified LF as potentially eradicable disease [71]. This was based on the fact that *W. bancrofti* did not have a natural reservoir outside humans. In addition, administration of antifilarials is effective at controlling transmission. Then in 2000, the World Health Organization (WHO) launched the Global Programme to Eliminate Lymphatic Filariasis (GPELF) [1, 52]. The initiative called for countries to map the geographic distribution of LF within their borders. After mapping was completed, countries were to implement mass drug administration (MDA) in at-risk populations and alleviating the suffering of patients with clinically significant manifestations. The WHO recommended that the MDAs treat infected individuals with DEC and IVM, either alone or in combination with albendazole. Recently, a study investigated the safety, efficacy, and pharmacokinetics of using a combination DEC, IVM, and albendazole together for treated *W. bancrofti*-infected patients (n=12) in Papua

New Guinea [72]. This study demonstrated that triple therapy provided long-lasting suppression of microfilaremia for 1 year in all patients and up to 2 years in 6 of the patients. Furthermore, there was a reduction in CFA after 1 year in the novel treatment group compared to participants receiving DEC and albendazole, which suggests that the triple therapy may be macrofilaricidal. Because of this research, the WHO has recommended that this triple therapy be adopted for the MDA.

At beginning of the GPELF, there were 81 countries endemic for LF with over 120 million people infected worldwide [73]. In 2010, reevaluation of countries with historically low LF prevalence led to their reclassification as nonendemic leaving only 73 countries. In the first 13 years of the campaign, around 5 billion treatments were given to people living in these endemic areas [1]. This has led to a drastic reduction in global LF prevalence from 120 million cases to 70 million cases. As of 2016, 20 endemic countries have reduced LF prevalence to levels believed to be unsustainable for disease transmission [1, 52, 73]. GPELF has made significant progress towards global elimination of LF, but there still remains a large population that is infected and an even larger population at risk for infection.

Limitations with the current treatment approach

The elimination strategy recommended by the WHO as part of the GPELF is limited. Current therapeutics target microfilariae, which means multiple treatment doses are required until the adult filarial worms clear. Interruptions in treatment can lead to repopulation of microfilariae by adult worms. The WHO recommends that the MDA must be implemented for at least 5 years [52], which places a heavy logistical burden on participating countries and runs the risk of

patient non-compliance [1]. Additionally, there are well-documented contraindications associated with antifilarial treatments in areas endemic to the subcutaneous filarial worms *Onchocerca volvulus* and *Loa loa*. DEC cannot be administered in onchocerciasis co-endemic areas because it quickly kills the microfilariae inducing a severe inflammatory response [74, 75]. This can lead to adverse systemic reactions such as skin lesions, fever, polyarthritides, and ocular reactions in patients with onchocerciasis as determined by Mf load [76, 77]. Similarly, DEC and IVM cannot be administered to areas co-endemic with loiasis due to an increased risk of severe neurologic events such as encephalopathy from rapid Mf death in the vasculature [78-80]. Unfortunately, 40% of LF-exposed populations live in sub-Saharan Africa where these infections are pervasive [81]. Considering these obstacles as well as the large population still infected, it is apparent that new therapeutics are needed. An effective and safe filarial vaccine, of which there is none, would overcome issues of noncompliance by providing long-lasting protection and improve the clinical outcomes of infected patients.

The need for a vaccine against lymphatic filariasis

Given the reasonable success of the GPELF in reducing worldwide prevalence of LF, some question the need for a vaccine. It is quite possible that global eradication can be attained with the triple therapy strategy. However, as stated earlier, current therapeutics do not permanently sterilize the adult worms. Therefore, interruptions in treatment risk repopulation of the Mf. Furthermore, an effective vaccine would address the growing concern that filariae may soon develop resistance to antifilarials [82]. Finally, there are few treatment options for

individuals living in areas co-endemic to onchocerciasis and loiasis due to contraindications with current drugs.

Limitations to developing a helminth vaccine

Filarial infection induces long-lasting, parasite-specific IgE responses in humans, which arms type 2 effector cells [83, 84]. This suggests that a vaccine consisting of filarial antigens might have the capacity to induce an allergic response in individuals who already produce helminth-specific IgE. Up until recently, *B. malayi* and *W. brancrofti* secreted venom allergen-like (VAL) were thought to be excellent vaccine candidates [85]. Administering filarial VAL in animals elicited a balanced Th1/Th2 response and IgG production that was protective [86]. However, a VAL hookworm vaccine (Na-ASP-2) failed human trials when researchers observed that the antigen induced urticaria in participants with pre-existing specific IgE [87] indicating that orthologs in other helminths would be unsafe to use as vaccine antigens.

Another limitation to developing a filarial vaccine is the lack of evidence for sterilizing immunity during natural infection. Still there is evidence that humans acquire partial immunity against infection. Endemic normals lack microfilaremia with no clinical disease despite living in endemic areas [35, 88]. As stated previously, these people are considered putatively immune, supporting the idea of acquired resistance. However, in many cases, diagnostics reveal the presence of CFA in many of these individuals [29]. So, it remains unclear whether endemic normals exhibit sterile or partial immunity, however, a Th1 response does appear important for establishing resistance [35].

Interestingly, other evidence for acquired immunity comes from the concept of concomitant immunity. It has been theorized that individuals infected with healthy, fecund adult filarial worms are resistant to further infection by L3s. In fact, Mf levels tend to level over time during chronic onchocerciasis, a subcutaneous filarial infection [89]. This suggests a mechanism exists that limits superinfection despite constant L3 exposure. Furthermore, patients infected with *W. bancrofti* adults exhibit elevated antibodies to L3s [90]. This also occurs in patients with *B. malayi* [91]. This stage-specific resistance may be due to immunomodulatory capacity of adult filarial worms and Mf [35], which impairs the immune response targeting these life-stages but leaves the L3 stage vulnerable. However, concomitant immunity may not be immunological, but rather due to secreted factors from adult worms or Mf that impair reinfection by L3s.

Vaccine studies have also provided evidence for acquired immunity to LF. To date, the vaccine method that imparts the greatest protection in animal models is immunization with irradiated L3s [15]. Immunizing Mongolian gerbils with irradiated *B. malayi* resulted in a 75% reduction in worm burden after subcutaneous challenge [92]. In another study, cows immunized with irradiated *Onchocerca ochengi* L3s, a filarial species transmitted by blackflies, had reduced adult worm burden of 64% against natural challenge and 100% against experimental challenge [93]. Further evidence for acquired immunity is that repeat infections of *B. pahangi* L3s in cats can impart partial resistance to re-infection, which mirrors human immunity after natural exposures [94]. Still, use of irradiated L3s would not be recommended for human use due to safety concerns.

Furthermore, given the challenge in obtaining parasitic material, it would be impractical to even try to develop a vaccine using live attenuated L3s.

Given the evidence for acquired resistance to infection, much of the filarial vaccine research has been focused on the antibody response in endemic populations with the hope of identifying an antigen(s) which could impart greater protection or possibly sterilizing immunity. Many studies have identified potential vaccine antigens based on the antibody response in endemic normals [88, 95-98]. These antigens have provided varying levels of protection but no sterilizing immunity. Studies have also investigated the antibody response of endemic populations against the body wall of filarial worms [99-104]. Indeed, studies showed sera from endemic populations are reactive against heavy myosin in the body wall of adult *B. malayi* females (*BmAF-Myo*). Vedi et al. demonstrated that jirds immunized with recombinant *BmAF-Myo* had a 70% reduction in Mf release and 55% reduction in number of live adult worms [99]. Finally, groups have evaluated excretory-secretory (ES) products released from filariae as potential vaccine antigens [105]. These products are derived from the worm cuticle or released from the secretory pore, vulva, anus, or mouth [106]. Protection has been demonstrated in various animal models using recombinantly expressed ES products [107-110]. Nag et al. showed that jirds immunized with recombinant translation initiation factor-1 of *Wolbachia* was an ES product that resulted in 70-72% reduction in Mf levels and 61-63% reduction in number of live adult worm [111].

To summarize, there is much evidence that acquired resistance to worm infection can occur, however, there is little to suggest that sterilizing immunity actually occurs during natural infection. Furthermore, vaccine studies evaluating antigens based on the antibody response in endemic normals have only demonstrated partial protection in animal models. Immunization with body wall antigens or ES products also only partially protected. The need for sterilizing immunity against filarial infections is a pressing issue. The adult worms can release upwards of 10,000 Mf in a day and are long-lasting, which means only a few adult worms are needed to maintain microfilaremia in humans [112].

Hidden antigens

Given the potential for a helminth antigen to induce an allergic reaction in endemic populations, helminth vaccine development has begun to focus on “hidden antigens” not typically exposed to the immune system. This idea of hidden antigens was first brought forward by Edward Munn [113]. He believed that helminth antigens exposed to the immune system would naturally elicit an immune response in sensitized populations. Therefore, vaccine development should focus on products that did not induce antibody response during natural infection. Furthermore, because these proteins are hidden from the immune system, then there would have been no evolutionary pressure for these proteins to evade the immune system. This could leave the proteins vulnerable to antibodies. One potential source of hidden antigens is the intestinal tract of helminths.

Targeting intestinal proteins in hookworm

Hookworm vaccine studies using intestinal *Necator americanus* antigens, aspartic protease-hemoglobinase (*Na*-APR-1) and glutathione S-transferase (*Na*-GST) were shown to induce the production of protective IgG [114-117]. Indeed, vaccinating dogs with *Na*-APR-1 reduced egg burden by 66.6% following challenge with *Ancylostoma caninum*, the hookworm species for canines. Another study showed that immunization with *Na*-GST reduced adult hookworm numbers by 32.2% in hamsters challenged subcutaneously with 250 *Necator americanus* L3 larvae. Not surprisingly, human vaccine trials using these antigens are underway [118]. Recently, phase 1 trials have been successfully completed using the GST vaccine [119]. The study showed that the vaccine induced a strong IgG response in immunized individuals. This result directly supports the hypothesis that intestinal proteins of helminth intestinal proteins act as hidden antigens.

Helminth feeding

Targeting intestinal proteins with drugs or antibodies requires the filariae to ingest material from the external environment. It is unclear whether adult filarial worms need a digestive tract for absorption of nutrients because these parasites feed on serum, which does not warrant complex digestion [120]. The life stages from microfilariae to L3 lack a functioning digestive tract [13]. In addition, studies have demonstrated that adult filarial worms can absorb nucleotides, amino acids, small peptides, sugars, and vitamins through their cuticle [121-124].

A previous study showed that *Litomosoides sigmodontis*, a filarial worm that infects cotton rats, can ingest hemoglobin [125]. Likewise, experiments soaking *Dirofilaria immitis* in FITC-labeled serum demonstrated entry of serum protein into

the intestine [126]. Therefore, it can be reasonably assumed that *B. malayi* adult worms can ingest material from the external environment.

Proteomic analysis of the intestinal tract of *B. malayi* adult worms

In a past study, our lab used proteomic analysis of the body wall, digestive tract, and intestine of *B. malayi* adult worms. The analysis identified a total of 4,785 proteins of which only 106 are enriched in the intestine and predicted to be localized to the luminal surface. [48]. These proteins had a diverse range of functions indicating the intestinal tract may serve a relevant role in filaria survival. For example, the intestinal tract exhibited a bias towards transporters and UDP transferases, suggesting that the tissue is involved in nutrient transport or waste disposal. Interestingly, myosin proteins were abundant in the digestive tract and were believed to be of non-myocyte origin because of the lack of musculature in the nematode intestinal tract. Given the importance of non-muscle myosin in regulating tight junctions and epithelial structure in mammals [127, 128], we believe that the digestive tract may function as a dynamic barrier. In addition, adhesion molecules containing an immunoglobulin-I set domain were found to be enriched supporting the idea that the intestine acts as a barrier.

Rationale for selection of candidate intestinal proteins

Based on the above study, we selected 9 intestinal proteins to investigate as potential therapeutic targets (Table 1). These proteins have low homology to humans while having high homology with other filarial species. None of the selected proteins are ES products. Furthermore, all 9 of these proteins are predicted to have an extracellular domain accessible to antibody binding. There is

previous evidence that antibody neutralization can facilitate worm clearance. Martin *et al.* demonstrated that in B-cell deficient mice infected with *Litomosoides sigmodontis*, a natural filarial species of cotton rats, vaccine-induced protection by administration of irradiated L3 larvae was abrogated [129]. Other groups have shown that loss of antibody production in mice enhanced survival of microfilariae and L3s [130]. Expanding on this, one group observed that mice vaccinated with *B. malayi* antigens produced a protective IgG antibody response [131]. The group later observed that in *Rhesus macaques* vaccinated with the same antigens, protection correlated well with IgG level [132]. In addition, endemic normals were shown to exhibit elevated IgG titers against the antigens. Similarly, another group reported elevated IgG titers to putatively protective carbohydrate antigens in endemic normals [35, 88]. As expected, the parasite killing ability by endemic normal sera is diminished when total IgG is depleted [131]. Data also indicates that IgA levels positively correlate to reduced worm burden and disease [133].

RNAi and FILARIAL WORMS

RNA interference (RNAi) is a natural pathway that silences gene expression by promoting mRNA degradation or translational inhibition. Small interfering RNA (siRNA) is able to utilize the pathway to repress gene expression by binding target mRNA. siRNA is double-stranded, non-coding RNA that ranges from 21 to 23 nucleotides in length with 2-nucleotide 3' overhangs [134]. Cells internalize the siRNA, which is then incorporated into the RNA-induced silencing complex (RISC). The molecular composition of the complex has yet to be fully elucidated, however, the minimal RISC sufficient for gene suppression consists of Argonaute 2 (Ago2),

Pub Locus	Gene Name	<u>H. sapiens</u>		<u>W. bancrofti</u>		<u>O. volvulus</u>		<u>L. loa</u>		<u>D. immitis</u>		Non-cytoplasmic Domain
		% Ident	Query Cov	% Ident	Query Cov	% Ident	Query Cov	% Ident	Query Cov	% Ident	Query Cov	
Cell Adhesion												
Bm1_39630	<i>Bma-lad-2</i>	28	45-1170	97	628-1171	87	26-1171	89	1-1171	82	1-1171	19-1120
Bm1_02820	<i>Bma-egf-like 02820</i>	35	53-206	96	1-269	82	3-269	89	62-269	79	2-269	1-225
Glycosylation/glucuronidation												
Bm1_44655	<i>Bma-fukutin</i>	31	138-364	96	1-362	73	1-364	85	1-364	75	1-364	28-364
Bm1_13480	<i>Bma-iugt</i>	27	35-509	95	1-425	29	214-293	81	1-423	72	155-502	1-486
Miscellaneous												
Bm1_48010	<i>Bma-egf-like 48010</i>	36	10-395	91	19-338	66	6-560	74	6-556	64	6-546	1-430
Proteases												
Bm1_38300	<i>Bma-peptidase</i>	28	136-586	90	90-819	67	1-819	74	1-819	70	1-819	81-1061
Bm1_53050	<i>Bma-reprolysin</i>	32	153-753	91	96-845	77	1-839	79	1-839	77	1-843	1-607
Possible Proteases												
Bm1_00205	<i>Bma-shrk</i>	27	54-161	80	142-229	52	70-227	46	126-264	55	110-264	26-264
Protease Inhibitors												
Bm1_09775	<i>Bma-serpin</i>	29	26-388	75	84-375	52	29-390	54	1-391	52	1-391	17-391

Table 1. List of Intestinal Proteins to be Evaluated as Therapeutic Targets

an endonuclease, with bound RNA. The RISC unwinds the RNA and preferentially incorporates the siRNA strand with the more thermodynamically unstable 5' end [135]. The passenger RNA strand is cleaved by Ago2 and degraded. The by Ago2 and degraded. The incorporated siRNA binds with perfect complementarity to target mRNA and Ago 2 cleaves the mRNA at the region corresponding to the 10 to 11 nucleotide positions of the siRNA [135, 136]. The degradation of the mRNA leads to decreased protein expression and can significantly affect cellular function.

RNAi provides an effective and specific means to identify essential filarial genes. Recently, the WHO has emphasized the importance of RNAi for advancing antifilarial drug discovery [137, 138]. Several groups have demonstrated gene silencing by siRNA leads to a decline in abundance of targeted transcript by 80-90% in adult filarial species [139-145]. The knockdown of corresponding protein typically results in reduced fecundity and viability of the worms. The duration of gene suppression has not been fully quantified, however, there are attempts to determine this using *ex vivo* transplantation experiments. Duration of knockdown will most likely depend on the filarial species, delivery method, protein half-life and target transcript abundance. Fortunately, in most cases, siRNA knockdown has persisted long enough to determine if a gene is critical for worm survival. Therefore, RNAi provides an established method for accomplishing the goal of this proposal, which is to identify essential intestinal proteins for adult filarial worms.

Hypothesis and specific aims

We hypothesize that the intestinal tract of *Brugia malayi* adult worms is essential for their survival. Identification of essential intestinal proteins may reveal novel vaccine targets. Even if these proteins are not effective as vaccine antigens, they could still be targetable by drugs. By evaluating FDA-approved drugs that inhibit the function of essential proteins in adult worms, we may discover new macrofilaricides that are easily translatable to human use. Finally, we postulate that intestinal proteins can act as “hidden antigens” that are not exposed to the immune system during natural infection. Filarial antigens can elicit an IgE response in infected individuals. This is a limitation when developing a vaccine because the antigen may induce an allergic reaction in endemic populations. We hypothesize that adult filarial intestinal antigens will not induce such a reaction, and therefore, could be safe to use in endemic areas.

In order to test these hypotheses, we employed the following specific aims:

Specific aim 1: Identification of essential intestinal proteins in adult filarial worms by siRNA knockdown

Specific aim 2: Determine whether filarial patient serum contains reactive IgE antibodies against these essential intestinal proteins

Chapter 2: Methods

METHODS FOR DATA AND EXPERIMENTS FOUND IN

CHAPTER 3

Parasites and culture

Female *B. malayi* adults used in this study were obtained from the NIH/NIAID Filariasis Research Reagent Resource Center (FR3) and TRS Laboratories in Athens, Georgia, USA. The worms were cultured in Dulbecco's Modified Eagle's Medium (Corning cellgro) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 1% L-glutamine (Sigma) for 24 hrs at 37°C in 5% CO₂ prior to siRNA treatment. Microfilariae (Mf) were obtained from adult female worms cultured *in vitro*.

siRNA Design

Using the BLOCK-iT™ RNAi Designer, we selected the top three *Bma*-iUGT siRNA duplexes for gene silencing activity and specificity. The *Bma*-iUGT siRNA and corresponding scrambled siRNA were synthesized by Life Technologies and purified by standard desalting methods. The 5'-3' sequences of the *Bma*-iUGT siRNA strands were as follows:

Bma-iUGT siRNA 1

sense: 5' GCCUAACGAAACUAAGCAAdTdT 3'

antisense: 5' UUGCUUAGUUUCGUUAGGCdTdT 3'

Bma-iUGT siRNA 2

sense: 5' GGCUUCCACAAUCUGAUUUdTdT 3'

antisense: 5' AAAUCAGAUUGUGGAAGCCdTdT 3'

Bma-iUGT siRNA 3

sense: 5' GGUGGUAUGAAUAGCAUAAdTdT 3'

antisense: 5' UUAUGCUAUUCAUACCACCCdTdT 3'

Demonstration of siRNA uptake and antibody ingestion by fluorescence microscopy

For demonstration of siRNA uptake, adult female worms were incubated in 5 μ M of 5' Cy3-labeled *Bma-iugt* siRNA 3 (Sigma-Aldrich) for 24 hrs. Adult worms incubated in media alone were used as a negative control. Both groups of worms were then stained with 10 μ g/mL of DAPI (Sigma-Aldrich) in PBS. Fluorescent images were captured by a Nikon Eclipse E600 fluorescent microscope and converged using NIS-Elements software. To demonstrate ingestion of antibody, adult female worms were incubated with 100 μ g of mouse Cy3-labeled IgG isotype control in 2 mL of culture media. The worms were imaged 24 hrs later using the TRITC filter on a Zeiss Axio Observer.A1.

siRNA incubation of *B. malayi* female adult worms

siRNA inhibition of *Bma*-iUGT in *B. malayi* adult female worms followed a protocol established by Aboobaker et al. with minor modifications [139]. For each timepoint, 5 adult female worms were soaked in an equal mixture of the *Bma*-iUGT siRNAs at a final concentration of 5 μ M in 850 μ L of culture media in a 5000 MWCO Pur-A-Lyzer™ dialysis tube (Sigma-Aldrich). This concentration of siRNA was

shown in multiple studies to be sufficient at silencing gene expression [139, 141, 142, 144]. The dialysis tubes were placed in 1 L beakers with 500 mL of culture media for 24 hrs at 37°C in 5% CO₂. Similarly, 5 adult female worms were soaked in media alone or scrambled siRNA (5 µM) in dialysis tubes for each timepoint as experimental controls. After the 24-hr incubation, the worms for each group were carefully extracted from the dialysis tubes and individually placed into wells with 1 mL of media. The worms were evaluated at timepoints 1, 3, and 6 days post-incubation for transcript knockdown, worm motility, MTT reduction, and microfilaria release.

Total RNA extraction and analysis of mRNA levels by RT-qPCR

Adult *B. malayi* female worms were homogenized in TRIzol (Thermo Fischer Scientific) after three freeze/thaw cycles using Matrix D lysis tubes (MP Biomedicals) agitated by a FastPrepTM-24 Biopulverizer (MP Biomedicals) for 7 minutes at 6 m/s. Chloroform was added to the homogenate, transferred to Phase Lock Gel tubes (5Prime), and phase separated at 11,900 g for 15 minutes at 4°C. The aqueous phase was collected and cold isopropanol was added to precipitate the RNA, which was then pelleted at 12,000 g for 1 hr and washed twice using 75% ethanol. The RNA pellet was resuspended in nuclease-free water and quantified using a NanoDrop 1000 (Thermo Fischer Scientific). cDNA was prepared using Superscript IV (Thermo Fischer Scientific) as per the manufacturer's protocol. The transcript expression levels of *Bma-iugt* and *B. malayi* house-keeping gene *gapdh* were assessed in duplicate 20 µL reactions using 1 µL of 20X TaqManTM gene expression assay (Thermo Fischer), 1 µL of

cDNA, and 18 μ L of TaqMan™ gene expression master mix (Applied Biosystems). PCR conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C cycle of 50°C with a 7500 Real-Time PCR System (Applied Biosystems). The primers used were as follows:

Bma-gapdh:

Forward primer: 5' TTGATCTCACTTGCCGACTC 3'

Reverse primer: 5' TGGTCTTCGGTGTATTCCAA 3'

Internal probe: 5' CAGCTAATGGACCGATGAAGGGGA 3'

Bma-iugt:

Forward primer: 5' TATCATTCGGCACCGTTACA 3'

Reverse primer: 5' ATTCATACCACCATGCGTCA 3'

Internal probe: 5' TCGCTGAGGGACGTCCAAACG 3'

Generation of rabbit polyclonal antibodies against *Bma*-iUGT peptides

Polyclonal anti-*Bma*-iUGT peptide antibodies were generated in New Zealand rabbits by Genscript using *Bma*-iUGT peptide sequences conjugated to keyhole limpet hemocyanin (KLH). The peptide sequences used were as follows: CYEKDEHLIAEGRPN, DSTGSKLAKTVKIDC, and CGQIANFDPYGRKMS. Cysteines were added at either the N- or C-terminus to facilitate KLH conjugation.

Western blot

B. malayi adult worms were incubated in 5 μ M combination of *Bma*-iUGT siRNA for 24 hrs using the previously mentioned method and transferred into individual wells with 1 mL of media. The adult worms were cultured for an additional 24 hrs and then homogenized in PBS (pH 7.4) and 4 μ L Halt™ Protease Inhibitor Cocktail (Thermo Scientific) using Matrix D lysis tubes (MP Biomedicals) agitated

by a FastPrepTM-24 Biopulverizer (MP Biomedicals) for 3 minutes at 4 m/s. Protein levels were quantified by the Bradford protein assay (Bio-Rad). For Western blot analysis, 10 µg of protein was separated on 10% Bis-Tris NuPAGE gel (Invitrogen) and blotted onto 0.2 µm nitrocellulose filter paper (Bio-Rad). After blocking overnight in 5% bovine serum albumin (BSA) in tris-buffered saline with 0.1% Tween 20 (TBS-T), the membrane was incubated with 1:4000 anti-UGT peptide antibodies (Genscript) and 1:1000 rabbit anti-β actin antibodies (Abcam) for 1 hr. Following this, the filter paper was washed with TBS-T and then incubated with 1:2000 horseradish peroxidase conjugated goat anti-rabbit IgG for 1 hr. The membrane subsequently washed and incubated in Chemiluminescent reagent, SuperSignalTM West Pico PLUS (Thermo Scientific), to visual the bands.

Worm motility

Worms were visualized with a dissecting microscope by an observer blinded to treatment category. Motility of the adult female *B. malayi* worms was rated based on the following scale 4 = active movement, 3 = modest reduction in movement, 2 = severe reduction in movement, 1 = twitching, and 0 = no movement.

MTT reduction

Metabolic function of the adult female worms was assessed by reduction of (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma using a protocol established by Comley et al [146]. For each group per timepoint, 2 worms were incubated in 0.5 mL of phosphate buffered solution (PBS) with 0.5 mg/mL of MTT for 30 minutes at 37°C in 5% CO₂. The worms were then transferred

into separate wells of a 96-well plate containing 200 μ L of DMSO and incubated at room temperature for 1 hr. MTT reduction was quantified by absorbance relative to a DMSO blank at 570 nm using a Synergy HTX multi-mode plate reader (BioTek).

Microfilaria (Mf) release

For each timepoint, adult worms were placed in new culture media 24 hrs prior to enumeration of microfilariae. After the overnight incubation, the worms were then removed for processing by the MTT reduction assay and RT-qPCR. The Mf in the well containing expended culture media (1 mL) were counted under a light microscope at high magnification.

In vitro culture with UGT inhibitors

Adult *B. malayi* worm incubation with UGT inhibitors

UGT inhibitors, sulfinpyrazone (ChemCruz) and probenecid (Invitrogen), were evaluated for macrofilaricidal activity *in vitro*. Sulfinpyrazone was resuspended in 1X PBS (pH 7.4) while probenecid was resuspended in deionized water. When testing sulfinpyrazone, adult *B. malayi* female worms were incubated in culture media with the drug for 8 days at concentrations of 2500 μ M, 1000 μ M, 200 μ M, 40 μ M, and 8 μ M. For probenecid, adult female worms were incubated in culture media with the drug for 7 days at concentrations of 5000 μ M, 2500 μ M, 1000 μ M, and 500 μ M. Worms were transferred into new media with corresponding drug concentrations every other day. As a negative control, worms were incubated

in culture media alone. Worm motility was scored using the previously mentioned scale for the course of the experiment.

***B. malayi* microfilariae incubation with UGT inhibitors**

The above UGT inhibitors were evaluated for microfilaricidal activity *in vitro*. For each drug, experiments were performed in triplicate at a concentration of 2×10^4 Mf/mL in culture media. Viability was determined by quantifying the number of motile larvae from 100 randomly selected Mf per well. The concentrations used for sulfinpyrazone were 2500 μ M and 200 μ M while the concentrations used for probenecid were 5000 μ M and 500 μ M. As a negative control, larvae were incubated in culture media alone

UGT Inhibitor Cytotoxicity Assay

We measured cytotoxicity of the UGT inhibitors using a Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific). HEK cells were seeded at 5×10^4 per well in DMEM (Quality Biological) with 10% Hyclone Cosmic Calf Serum (Thermo Fischer), 200 μ M of L-glutamine (Quality Biological), and 50 μ g/mL of gentamicin (Quality Biological) at 37°C in 5% CO₂. We then incubated the cells with various concentrations of the UGT inhibitors overnight. Following this, we transferred 50 μ L of media from each well to a new 96-well plate and then added 50 μ L of reaction buffer. We incubated the mixture for 30 minutes and then added 50 μ L of stop solution. We measured absorbance at 490 nm and 680 nm. We employed the following controls: a spontaneous LDH activity control which was incubated with the vehicle only and a maximum LDH activity control which was incubated with

nothing but later lysed prior to incubation with the reaction buffer. We calculated absorbance for each well by subtracting the 680 nm absorbance value (background) from the 490 nm absorbance value. We then calculated percent cytotoxicity using the following equation:

$$(1) \quad \% \text{ Cytotoxicity} = \frac{(\text{UGT inhibitor LDH activity} - \text{Spontaneous LDH activity})}{(\text{Maximum LDH activity} - \text{Spontaneous LDH activity})}$$

Generation of Ruc-antigen fusion proteins

Bma-iUGT was expressed as a *Renilla reniformis* luciferase (Ruc) fusion protein by cloning the *Bma*-iUGT coding sequence in *pREN2* (Genscript). The *Bma*-iUGT signal sequence as predicted by signalP was removed prior to synthesis. Plasmid encoding the fusion protein was used to transform TOP10 cells (Thermo Fischer) and plasmid DNA was obtained from colonies selected on Kanamycin (50 µg/ml) as per the manufacturer's guidelines (Qiagen Midi-Prep). 293F cells grown in 293 Freestyle Medium as suspension cultures were transfected with 30 µg of *Bma*-iUGT-Ruc plasmid, at final concentration of 1 µg per 1 x 10⁶ cells (Thermo Fischer Scientific) per mL, and cultured at 37°C with 8%CO₂ on a rotary shaker at 125 rpm. After 72hrs, the cells were pelleted and sonicated in LIPS lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1% TritonX-100, 50% glycerol, protease inhibitors (Mini from Roche)). The lysate was centrifuged to remove cellular debris and supernatant containing the *Bma*-iUGT-Ruc fusion proteins were stored at -80°C for later use.

Luciferase immunoprecipitation system

Antibody titers were measured using a luciferase immunoprecipitation system (LIPS) assay [147-149]. For IgG and IgE quantification, serum was diluted to 1:100 and 1:10 respectively in 50 μ L of LIPS master mix (20 mM Tris pH 7.5, 100 mM NaCl, 5mM MgCl₂, 1% Triton X-100) and mixed with 50 μ L of the UGT-Ruc fusion containing 1×10^6 light units (LU) of protein in PBST (PBS with 0.05% Tween-20). The reaction mixture was incubated in a 96-well polypropylene plate for 10 minutes at room temperature and transferred to a 96-well high throughput screening filter plate (Milipore) containing 5 μ L of a 50% suspension of Ultralink protein A/G (Pierce) or Ultralink anti-human IgE beads in PBS and incubated for an additional 15 minutes at room temperature. The plates were washed under vacuum with 200 μ L of LIPS master 3x followed by PBS once. The relative light units (RLU) were measured with a Berthold LB 960 Centro microplate luminometer with 50 μ L of coelenterazine solution (Promega). For these experiments, samples were run in duplicate, and the calculated RLU was adjusted for the measured RLU of UGT fusion protein without serum.

Statistical analysis

The siRNA and UGT inhibitor experiments were repeated two times under similar conditions. Data shown for these experiments is of the repeat. For the siRNA experiments, data was analyzed using one-way analysis of variance (ANOVA) or T-test by PRISM 7.0. Following ANOVA, individual comparisons of mean values were performed using Tukey's multiple comparisons test. For the UGT inhibitor experiments, we performed AUC analysis followed by one-way ANOVA to determine significance. Statistical significance between the

experimental and control groups was designated as follows: * for p values <0.05, ** for p values <0.01, and *** for p values <0.001.

METHODS FOR DATA AND EXPERIMENTS FOUND IN CHAPTER 4

Parasites and culture

This study used *B. malayi* female adults obtained from the NIH/NIAID Filariasis Research Reagent Resource Center (FR3) and TRS Laboratories in Athens, Georgia, USA. Before siRNA inhibition, the adult worms were incubated in Dulbecco's Modified Eagle's Medium (Corning cellgro) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 1% L-glutamine (Sigma) for 24 hrs at 37°C in 5% CO₂.

siRNA Design

We employed the BLOCK-iT™ RNAi Designer when selecting the siRNA duplexes for gene silencing activity and specificity. The siRNA sequence with the greatest probability of success was selected for each target, and for some targets, multiple sequences were selected to best ensure knockdown. Life Technologies synthesized the target-specific siRNA (*Bma-lad-2*, *Bma-fukutin*, *Bma-shhk*, *Bma-serpin*) and purified the complexes by standard desalting methods. Similarly, other target-specific siRNAs (*Bma-egf-like-02820*, *Bma-egf-like-48010*, *Bma-peptidase*,

Bma-reprolysin) were obtained through Dharmacon. The 5'-3' siRNA sequences used in this experiment are as follows:

Bma-LAD-2 siRNA 1

sense: 5' GCAAGUACUACCAUACUAUdTdT 3'
antisense: 5' AUAAGUUGGAAUUCGUUGCdTdT 3'

Bma-LAD-2 siRNA 2

sense: 5' GCGCAUAUCGCAAGUAAAUdTdT 3'
antisense: 5' AUUUACUUGCGAUAUGCGCdTdT 3'

Bma-LAD-2 siRNA 3

sense: 5' GCGAAUAGUCGAUACCUAAdTdT 3'
antisense: 5' UUAGGUAUCGACUAUUCGCdTdT 3'

Bma-Fukutin siRNA 1

sense: 5' CCACCCATTTCGCAGATTT 3'
antisense: 5' AAAUCUGCGAAAUGGGUGGdTdT 3'

Bma-Fukutin siRNA 2

sense: 5' GGAGCGAGAGTGAATGGAAdTdT 3'
antisense: 5' UUCCAUUCACUCUCGCUCCdTdT 3'

Bma-Fukutin siRNA 3

sense: 5' GCTAACGTTGCAAATTATTdTdT 3'
antisense: 5' AAUAAUUUGCAACGUUAGCdTdT 3'

Bma-ShTK siRNA 1

sense: 5' GCGCCTTCTACAGCAGTAAdTdT 3'
antisense: 5' GCGCCUUCUACAGCAGUAAdTdT 3'

Bma-ShTK siRNA 2

sense: 5' GGUGGUAUGAAUAGCAUAAdTdT 3'
antisense: 5' UUAUGCUAUUCAUACCACCdTdT 3'

Bma-ShTK siRNA 3

sense: 5' GCUAAAGAACUAUGCGCUAdTdT 3'

antisense: 5' UAGCGCAUAGUUCUUUAGCdTdT 3'

Bma-Serpina siRNA

sense: 5' GGAUUUCGAGUGAGACAAAdTdT 3'

antisense: 5' UUUGUCUCACUCGAAAUCCdTdT 3'

Bma-EGF-like-02820 siRNA

sense: 5' GUAUCGAGGGCAAGGGAAAdTdT 3'

antisense: 5' UUUCCCUUGCCCUCGAUACdTdT 3'

Bma-EGF-like-48010 siRNA

sense: 5' GCAACAAAUGCAAGAAUAdTdT 3'

antisense: 5' UUAUUCUUGCAUUUGUUGCdTdT 3'

Bma-Peptidase siRNA 1

sense: 5' AGGAAAGGUUGUUAGGAUAdTdT 3'

antisense: 5' UAUCCUAACAACCUUCCUdTdT 3'

Bma-Reprolysin siRNA 3

sense: 5' GGAUAAUGUGAAAGGAAUAdTdT 3'

antisense: 5' UAUUCCUUUCACAUUAUCCdTdT 3'

Cy3-labeled siRNA treatment and fluorescence microscopy

Adult female worms were treated with 5 μ M of 5' Cy3-labeled *Bma*-LAD-2 siRNA 1 (Sigma-Aldrich) for 24 hrs to demonstrate uptake of siRNA into the intestinal tract. For the negative control, the worms were cultured in media alone were used as a negative control. As a counterstain, the samples were treated with 10 μ g/mL of 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich)

in PBS. Imaging was taken with a Nikon Eclipse E600 fluorescent microscope and converged by NIS-Elements software.

siRNA treatment of *B. malayi* female adult worms

For siRNA inhibition of the target gene expression, we soaked the *B. malayi* adult female worms in culture media with siRNA slightly modifying a previously established protocol [139]. For each timepoint, we incubated 5 adult female worms for 24 hrs in an equal mixture of the siRNAs (*Bma*-LAD-2, *Bma*-Fukutin, *Bma*-ShTK, *Bma*-Serpin) or one siRNA (*Bma*-EGF-like-02820, *Bma*-EGF-like-48010, *Bma*-Peptidase, *Bma*-Reprolysin) at a total concentration of 5 μ M in 850 μ L of media in a 5000 MWCO Pur-A-Lyzer™ dialysis tube (Sigma-Aldrich). Previous studies have shown that this concentration of siRNA sufficiently silences gene expression [139, 141, 142, 144]. We placed the dialysis tubes in a beaker with 500 mL of culture media at 37°C in 5% CO₂. For the control groups, 5 adult female worms were incubated alone in media or with scrambled siRNA (5 μ M) under conditions similar to the target siRNA-treated group. The worms were extracted after the 24-hr incubation and placed individually into 1 mL of culture media. Initially, worms were evaluated 1-day post-incubation for transcript knockdown, worm motility, MTT reduction, and microfilaria release. For *Bma-lad-2*, we later evaluated the worms 6 days post-siRNA incubation.

Total RNA extraction and analysis of mRNA levels by RT-qPCR

Adult *B. malayi* female worms were treated with TRIzol (Thermo Fischer Scientific) and subjected to three freeze/thaw cycles. The adult filariae were placed

in Matrix D lysis tubes (MP Biomedicals) and homogenized by a FastPrep™-24 Biopulverizer (MP Biomedicals) for 7 minutes at 6 m/s. We added chloroform to the homogenate and phase separated the mixtures in Phase Lock Gel tubes (5Prime) at 11,900 g for 15 minutes at 4°C. After the top layer (aqueous phase) was collected, we precipitated the RNA using cold isopropanol and then pelleted it at 12,000 g for 1 hr. The RNA pellet was washed twice using cold 75% ethanol. We resuspended the RNA in nuclease-free water and quantified the sample concentrations using a NanoDrop 1000 (Thermo Fischer Scientific). Using Superscript IV (Thermo Fischer Scientific), we synthesized cDNA from mRNA per the manufacturer's protocol. We quantified target gene and *B. malayi* house-keeping gene *gapdh* expression levels in duplicate 20 µL reactions using 1 µL of 20X TaqMan™ gene expression assay (Thermo Fischer), 1 µL of cDNA, and 18 µL of TaqMan™ gene expression master mix (Applied Biosystems). We employed the following PCR conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C, and 1 min at 60°C cycle of 50°C with a 7500 Real-Time PCR System (Applied Biosystems). The following Taqman primer and internal probes were used:

Bma-gapdh:

Forward primer: 5' TTGATCTCACTTGCCGACTC 3'

Reverse primer: 5' TGGTCTTCGGTGTATTCCAA 3'

Internal probe: 5' CAGCTAATGGACCGATGAAGGGGA 3'

Bma-lad-2:

Forward primer: 5' GTGATCCACGGCTTACGATT 3'

Reverse primer: 5' CAGGCACATCAAGCACAGTT 3'

Internal probe: 5' TGCTCGTGGCTTTCATTTCAGGA 3'

Bma-futkin:

Forward primer: 5' AGGTTATTTTCATGTGCCCTGC 3'

Reverse primer: 5' ATTCCATTCACTCTCGCTCCA 3'

Internal probe: 5' AGGCGGATTACGGTAATTGGCGAGT 3'

Bma-shtk:

Forward primer: 5' TGCACTGATCCAATGGCAAA 3'

Reverse primer: 5' GTTACTGCTGTAGAAGGCGC 3'

Internal probe: 5' TCGGCCAAAACATGTGGATTTTGC GG 3'

Bma-serpin:

Forward primer: 5' ACGTGCGCAGTTAGACTTTG 3'

Reverse primer: 5' GCCTCTGCGATATAAGCCAA 3'

Internal probe: 5' GCGGACGGTGAAACGAAGCAGCA 3'

Bma-egf-like-02820:

Forward primer: 5' GCTTACACGGTGGCAGAAAA 3'

Reverse primer: 5' AAGCCACCTATCTGCTCTCC 3'

Internal probe: 5' TCGAGGGCAAGGGAAACTGGAA 3'

Bma-egf-like-48010:

Forward primer: 5' ACCTGGCTTCATGGGAGAAA 3'

Reverse primer: 5' CTTCAACACAGTCGCAAACA 3'

Internal probe: 5' TGCTGCCGGTCTTATGGGCG 3'

Bma-peptidase:

Forward primer: 5' CAGCCATTATTGGCCAGGAC 3'

Reverse primer: 5' AAATGAAGTGGTGCCGCATT 3'

Internal probe: 5' AGCCTTCCAACCTTGGTTCATCCCAACA 3'

Bma-reprolysin:

Forward primer: 5' TGGAACACAGTGATCAGGCT 3'

Reverse primer: 5' AACGGCATTCCACTTATCG 3'

Internal probe: 5' CCCATTTTCGTGTGCAATAGTTGCAGCA 3'

Generation anti-*Bma*-LAD-2 polyclonal antibodies

For the Western blot analysis and LIPS assay, polyclonal anti-*Bma*-LAD-2 peptide antibodies were generated by Genscript. Rabbits were immunized with *Bma*-LAD-2 peptide sequences conjugated to keyhole limpet hemocyanin (KLH). The peptide sequences used are as follows: CYEKDEHLIAEGRPN, DSTGSKLAKTVKIDC, and CGQIANFDPYGRKMS. To facilitate binding to KLH, cysteines were added at either the N- or C-terminus of the peptides.

Western blot analysis of *Bma*-LAD-2

Prior to Western blot analysis, we incubated the *B. malayi* adult worms in 5 μ M combination of *Bma*-LAD-2 siRNA for 24 hrs followed by an additional 24 hr culture in fresh media. The adult filariae were transferred into Matrix D lysis tubes (MP Biomedicals) with PBS (pH 7.4) and 4 μ L of Halt™ Protease Inhibitor Cocktail (Thermo Scientific) then homogenized using a FastPrep™-24 Biopulverizer (MP Biomedicals) for 3 min at 4 m/s. Protein concentration was quantified by Bradford protein assay (Bio-Rad). Protein lysate (10 μ g) was separated on 10% Bis-Tris NuPAGE gel (Invitrogen) and then transferred onto a 0.2 μ m nitrocellulose filter paper (Bio-Rad). The filter paper was blocked overnight in 5% bovine serum albumin (BSA) in tris-buffered saline with 0.1% Tween 20 (TBS-T). After the overnight blocking, the membrane was incubated with 1:7000 anti-LAD-2 peptide antibodies (Genscript) and 1:1000 rabbit anti- β actin antibodies (Abcam) for 1 hr. The membrane was washed three times with TBS-T for 15 min. Horseradish peroxidase conjugated goat anti-rabbit IgG antibody was incubated for 1 hr with the filter paper at a dilution of 1:2000. After washing again with TBS-T, the

membrane was developed with Chemiluminescent reagent, SuperSignal™ West Pico PLUS (Thermo Scientific).

Evaluation of worm motility

Motility was evaluated based on the following scale 4 = active movement, 3 = modest reduction in movement, 2 = severe reduction in movement, 1 = twitching, and 0 = no movement. A blinded observer rated worm motility for each group under a dissecting microscope.

Measuring MTT reduction

Metabolic function was evaluated using a (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay from Sigma [146]. For each group per timepoint, 2 worms were treated with 0.5 mg/mL of MTT in 0.5 mL of PBS for 30 minutes at 37°C in 5% CO₂. Each worm was then transferred into a well containing 200 µL of DMSO of a 96-well plate at room temperature for 1 hr. Quantification of MTT reduction was measured based on absorbance relative to a DMSO blank at 570 nm with a Synergy HTX multi-mode plate reader (BioTek).

Quantifying microfilaria (Mf) release

Prior to quantifying Mf release, we incubated the adult worms in 1 mL of fresh media for 24 hrs. The adult filariae were then removed for evaluation by the MTT reduction assay and RT-qPCR. The Mf were enumerated under a light microscope at high magnification in the wells containing expended media.

Transmission electron microscopy

B. malayi female worms were treated *Bma*-LAD-2 siRNA for 24 hrs and then cultured for an additional 48 hrs. For morphological evaluation, the female filaria were first washed in PBS (pH 7.4) and then fixed with 2.5% paraformaldehyde, 1% glutaraldehyde in 0.12 M Millong's phosphate buffer (pH 7.4) overnight at room temperature. Following this step, the samples were post-fixed with 1% osmium tetroxide in 0.12 M Millong's phosphate buffer (pH 7.4) for 100 min then fixed en bloc with 2% aqueous uranyl acetate for 90 min. The samples were dehydrated in graded ethanol solutions (75% to 100%) for 10 min each. The worms were embedded in low viscosity epoxy resin (modified Spurr's recipe) and then dried at 70°C overnight. Ultrathin sections (75 nm) were cut on a Reichert Ultracut E Ultramicrotome and then stained with 0.2% lead citrate. Reagents used were obtained from Electron Microscopy Services. Samples were visualized using a Hitachi HT7700 Transmission Electron Microscope at an accelerating voltage of 80 kV.

Ruc-antigen fusion protein

The *Bma*-LAD-2-*Renilla reniformis* luciferase (Ruc) construct was inserted into a pREN2 vector by Genscript. The predicted signal sequence was removed prior to gene synthesis. The vector was cloned into TOP10 cells (Thermofischer) and amplified on agarose plates with kanamycin 50 µg/mL. Plasmid DNA was isolated and purified using a Midiprep kit (Qiagen) per the manufacturers guidelines. 293F cells (Thermofischer Scientific) were transfected with 30 µg of *Bma*-LAD-2 plasmid at a concentration of 1×10^6 cells per mL. The 293F cells

were collected 72 hrs later and homogenized. The lysate was stored at -80°C for later use.

Luciferase immunoprecipitation system (LIPS)

We employed the LIPS assay to measure antibody titers in serum from filarial patients [147-149]. In a 96-well polypropylene plate, serum was diluted to 1:100 for IgG and 1:10 for IgE in 50 μ L of LIPS master mix (20 mM Tris pH 7.5, 100 mM NaCl, 5mM MgCl₂, 1% Triton X-100) with PBS-T added to bring the volume to 100 μ L. We added 1×10^6 light units (LU) of the LAD-2-Ruc fusion protein to the mixture and incubated it at room temperature for 10 min. Purification of the antigen-antibody complex involved adding 5 μ L of a 50% suspension of Ultralink protein A/G (Pierce) or Ultralink anti-human IgE beads in PBS to a 96-well filter plate (Milipore) and then applying a vacuum. The serum mixture was then added and allowed to incubate for 15 minutes at room temperature. A vacuum was applied to filter plate leaving only antigen-antibody complexed bound to beads in the wells. The samples were washed with 200 μ L of LIPS master mix thrice and with PBS once. Using Berthold LB 960 Centro microplate luminometer, emitted LUs Were measured after addition of 50 μ L of coelenterzine solution (Promega) to each sample well. The serum samples were run in duplicate, and the calculated RLU was the emitted RLU for only the LAD-2 fusion protein subtracted from the emitted RLU for each sample.

Statistical analysis

The siRNA experiments for *Bma*-LAD-2 were repeated twice under the same conditions. Data shown for these experiments is of the repeat. All other siRNA experiments were only performed once. The worm motility and Mf release data was analyzed by one-way analysis of variance (ANOVA) using statistical package in PRISM 7.0. Validity of the one-way ANOVA was verified by performing individual comparisons of mean values using Tukey's multiple comparisons test. For the gene expression and MTT reduction data, a T-test was used to determine significance. The p values for each experimental and control group was designated as follows: * for p values <0.05, ** for p values <0.01, and *** for p values <0.001.

Chapter 3: Evaluating an intestinal UDP-glucuronosyltransferase in *Brugia malayi* adult worms as a potential therapeutic target

INTRODUCTION

Lymphatic filariasis (LF) is a debilitating disease caused by the tissue-invasive nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* with 70 million people infected worldwide and over a billion people at risk for infection [1]. Since 2000, the Global Programme to Eliminate Lymphatic Filariasis has substantially reduced the number of people infected or at risk for infection. However, it has become apparent that new strategies must be implemented in order to attain global eradication of LF [150, 151].

Development of new therapeutics that target adult filarial worms would greatly enhance our ability to eliminate lymphatic filariasis. When given individually, the antifilarial drugs diethylcarbamazine (DEC), ivermectin (IVM), and albendazole are effective against the microfilaria (Mf) stage but exhibit little activity against adult filarial worms [152]. Use of all three medications together appear to have a macrofilaricidal effect, but due to the adverse effects caused by their potent microfilaricidal activity DEC and ivermectin cannot be used for mass drug administration in areas endemic for *Loa loa* or *Onchocerca volvulus*. Therefore,

development of a short-course macrofilaricidal agent or a vaccine would be very helpful for eradication efforts.

Unlike cestodes and trematodes, nematodes have a complete intestinal tract. Over the past 15 years, intestinal proteins of hookworms and barber pole worms have been shown to be effective vaccine candidates in animal models [114-117, 153, 154]. Considering this work, our lab performed a proteomic analysis of the intestine, body wall, and reproductive tract of adult *Brugia malayi* worms to potentially identify novel drug and vaccine targets for filariasis [48]. We identified 106 proteins that are enriched in the intestinal gut of the adult worms. Of these intestinal proteins, we selected a subset for evaluation as drug and vaccine candidates based on high homology with other filarial species, extracellular domains with accessibility to drugs and antibody, and predicted function.

In this study, an adult *B. malayi* intestinal protein, UDP-glucuronosyltransferase (*Bma*-iUGT, Bm1_13480), was identified as a potential therapeutic target. We determined that this protein was essential for worm survival using small interfering RNA (siRNA) to knockdown expression. Importantly, we identified two FDA-approved commercially available UGT inhibitors that exhibit macrofilaricidal activity *in vitro*. Finally, we analyzed the antibody response in filarial patients against *Bma*-iUGT and found that neither infected individuals nor endemic normal develop detectable levels of IgE against *Bma*-iUGT, suggesting it would not induce allergic reactions if used in a vaccine.

RESULTS

***B. malayi* intestinal UGT exhibits high homology to other filarial species**

Previously, we reported that *Bma*-iUGT was a protein preferentially expressed in the intestine of *B. malayi* adult worms [48]. Upon Basic Local Alignment Search Tool (BLAST) analysis, we found the protein to have high sequence homology ($\geq 75\%$) with other filarial species and low homology ($< 45\%$) to humans. Predictive analysis using the InterPro database revealed that the protein contains a large lumenally-expressed domain likely accessible to small molecules or ingested antibodies. In this paper, we expanded our analysis to include other helminth species, *Ancylostoma duodenale* (hook worm) and *Haemonchus contortus* (barber pole worm) as well as *Caenorhabditis elegans*, a free-living model for parasitic nematodes. Given the high predicted homology of *Bma*-iUGT between *B. malayi* and *Dirofilaria immitis*, we also evaluated orthologs in cat and dogs. We generated a phylogenetic tree (Fig 3) by first aligning the *Bma*-iUGT peptide sequences using Multiple Sequence Comparison by Log-Expectation (MUSCLE) and then creating a tree based on efficient maximum-likelihood estimation method by the LG model. As expected, we see a high level of relatedness between *Bma*-iUGT and filarial orthologs, including *D. immitis*. Importantly, there is significant evolutionary distance between this UGT and orthologs in humans, cats, and dogs.

***Brugia malayi* iUGT expression is stage specific**

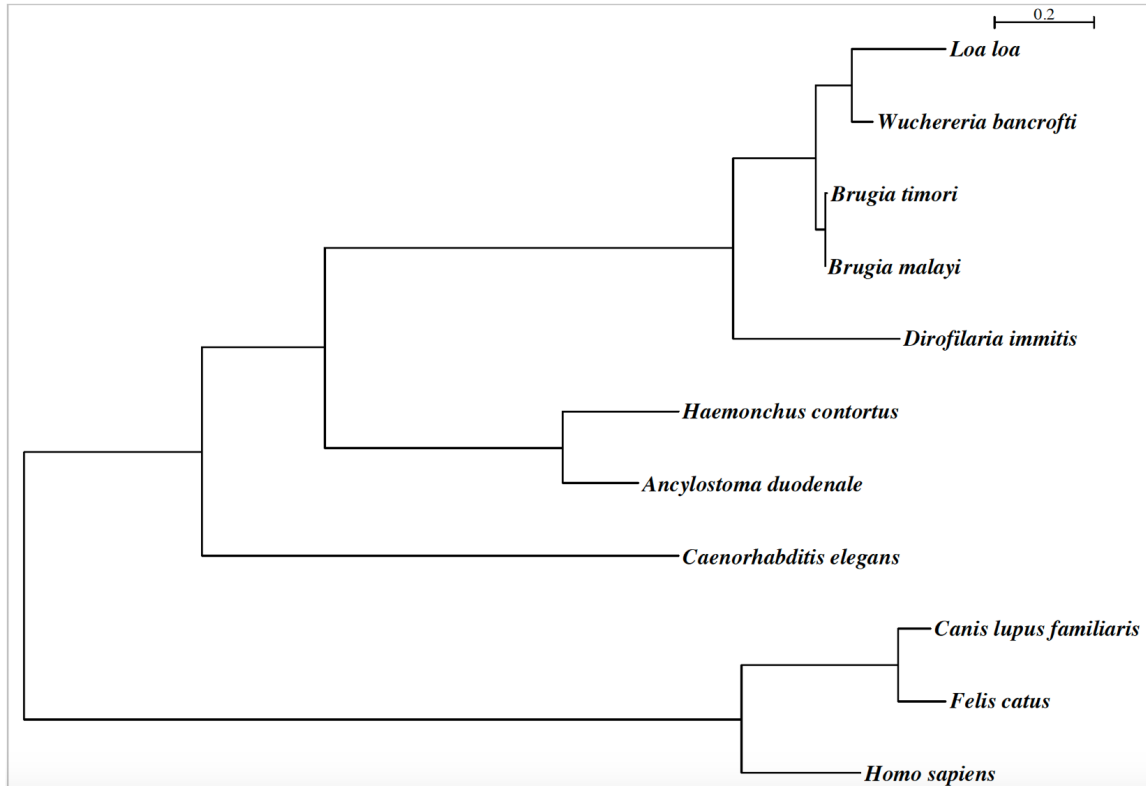


Figure 3. Phylogenetic comparison of *Bma*-iUGT.

Based on *B. malayi* cDNA sequence alignment, there is a high level of relatedness to other filarial species for *Bma*-iUGT and vast evolutionary distance to cats, dogs, and humans. The sequence alignment was generated using MULTiple Sequence Comparison by Log-Expectation (MUSCLE) performed by software SeaView. The tree was constructed based on the maximum likelihood method. The phylogenetic scale indicates genetic change as measured by the number of nucleotide substitutions per site

Previous studies have demonstrated that *Bma*-iUGT is not expressed in all the larval stages. Li et al. observed that *Bma-iugt* transcript expression occurred only in the adult female and male worm stage as well as the larval stage 3 (Table 2) [155]. As expected, *Bma*-iUGT protein expression was determined to be specific to these stages as well [156].

Uptake of target specific siRNA in the intestinal tract of adult *B. malayi* worms

To evaluate whether siRNA reaches the intestinal tracts of adult *B. malayi* worms, Cy3-conjugated *Bma*-iUGT siRNA was added to the culture media of adult *B. malayi* worms for 24 hrs. Visualization of the adult worms showed clear uptake of siRNA throughout the intestinal tract (Fig 4A-B). In contrast, imaging at the same exposure time revealed no apparent signal in the intestine of adult *B. malayi* cultured in media alone (Fig 4C-D).

***Bma*-iUGT siRNA reduces target transcript and protein expression**

We next evaluated reduction in target transcript and protein levels. We selected timepoints of 1, 3, and 6 days post-siRNA treatment based on a protein half-life of approximately 10 hrs for UDP-glucuronosyltransferases (UGT) [157]. After a 24-hr incubation with target-specific siRNA or scrambled siRNA, we compared *Bma-iugt* mRNA expression between the treated worms relative to the media control by RT-qPCR (Fig 5A). Transcript expression was normalized employing the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase*

	Adult Male	Adult Female	L3 Larvae	MF	Immature MF
<i>Bma-iugt</i> transcript	X	X	X	—	—
<i>Bma-iUGT</i> protein	X	X	X	—	—

Table 2. Stage specific analysis of *Bma-iUGT* in *B. malayi*

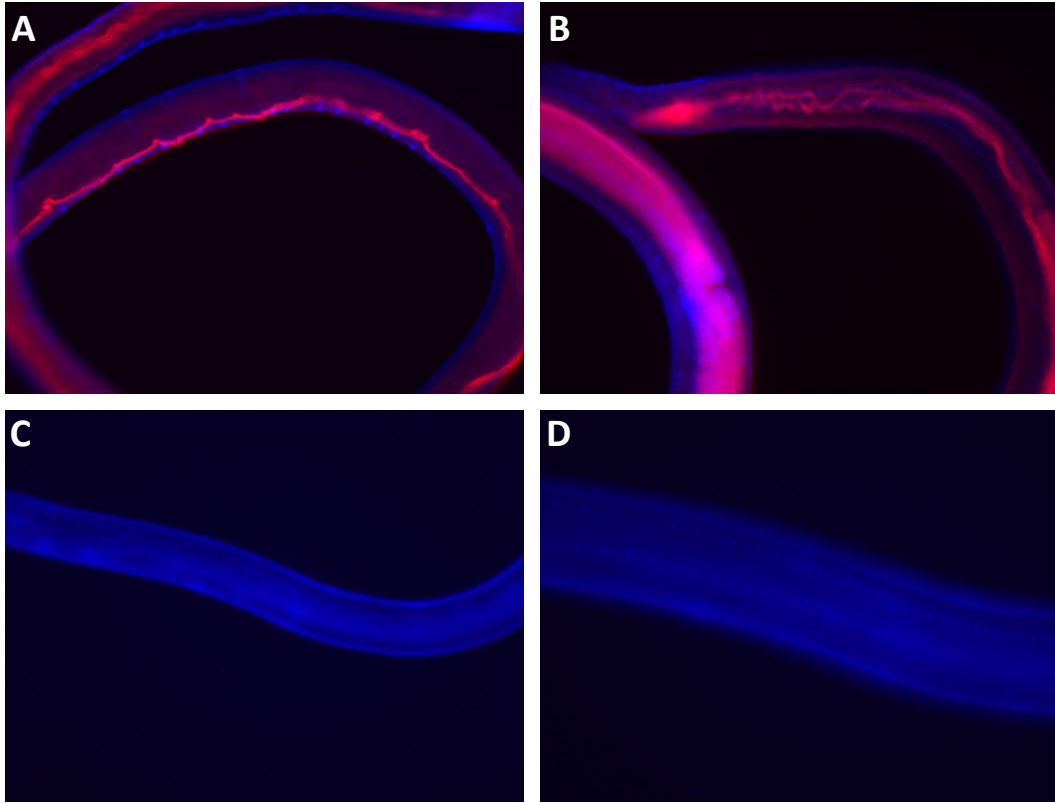


Figure 4. Cy3-labeled *Bma*-iUGT-specific siRNA enters the intestinal tracts of female *B. malayi* adult worms.

B. malayi adult worms incubated with Cy3-labeled siRNA (red) for 24 hrs and visualized at magnifications of (A) 100x and (B) 200x. Worms incubated in media alone for 24 hrs and visualized at magnifications of (C) 100x and (D) 200x. Worms were counterstained with DAPI (blue).

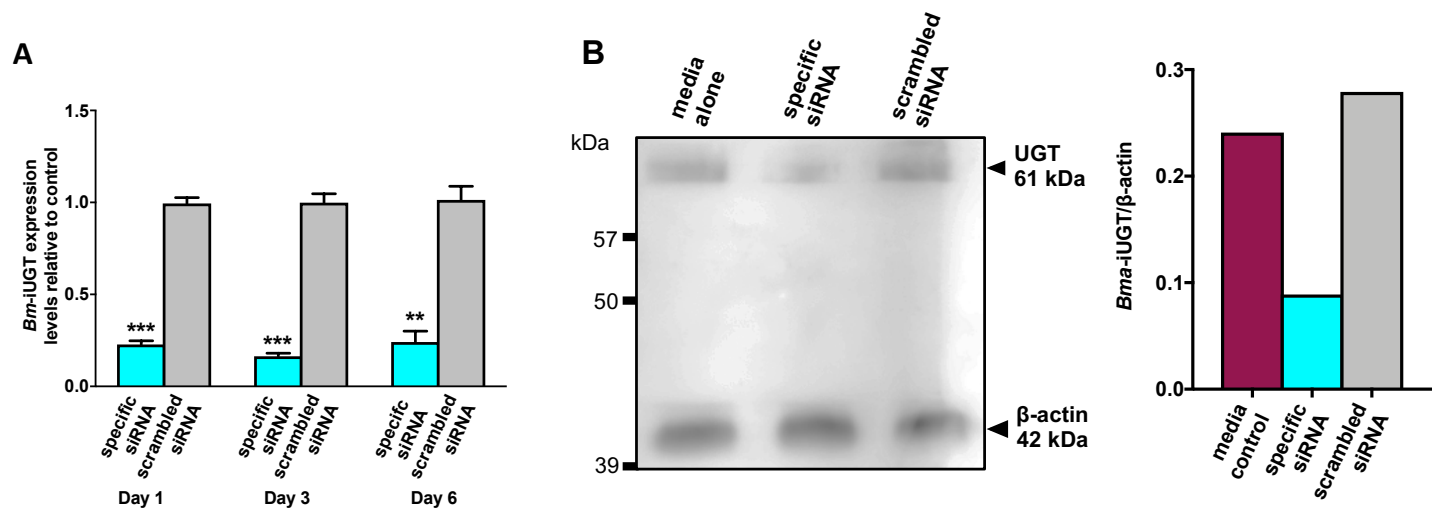


Figure 5. *Bma-iUGT* siRNA incubation reduces target transcript and protein levels in adult *B. malayi* worms.

Adult female worms were incubated with *Bma-iUGT* siRNA, scrambled siRNA, or media alone. (A) Target mRNA levels in the specific siRNA and scrambled siRNA treated groups were measured by RT-qPCR relative to the media control normalized to *Bma-gapdh*. (B) *Bma-iUGT* expression was evaluated 24 hrs post-siRNA incubation using anti-*Bma-iUGT* peptide antibodies. * for p values <0.05, ** for p values <0.01, and *** for p values <0.001

(*Bma-gapdh*, *Bm5699*). *Bma*-iUGT siRNA treatment resulted in a 77.1% reduction ($p=.0056$) in target mRNA compared to the controls 1-day post-siRNA treatment. Transcript knockdown was sustained throughout the experiment with a 76.23% decrease ($p=.0003$) in *Bma-iugt* transcription 6 days post-siRNA incubation. As expected, there was no significant difference in target transcription between the media control and scrambled siRNA groups.

Bma-iUGT knockdown was further substantiated by Western blot analysis (Fig 5B). Target protein expression was evaluated 24 hrs post-siRNA incubation using anti-*Bma*-iUGT peptide antibodies. There was a robust reduction in UGT expression with the *Bma*-iUGT siRNA treated worms compared to controls normalized to β -actin (87% reduction in *Bma*-iUGT/ β -actin)

***Bma*-iUGT knockdown results in decreased worm viability and fecundity**

After successfully demonstrating that siRNA reduces *Bma*-iUGT expression, we evaluated for any resultant changes in worm motility, Mf release, and metabolism. Adult worm motility was scored on a scale from 0 to 4, with 0 indicating no movement and 4 indicating active movement. At day 1 post-siRNA incubation, we observed a 77.1% reduction in motility with the *Bma*-iUGT siRNA-treated group compared to the scrambled control ($p=.0006$, Fig 6A). This dramatic reduction in motility was maintained through day 6 (78.94% reduction, $p=.0004$).

We also observed a dramatic decrease in Mf release per adult worm per 24-hr period after *Bma*-iUGT knockdown. At day 1, there was a 62.52% reduction

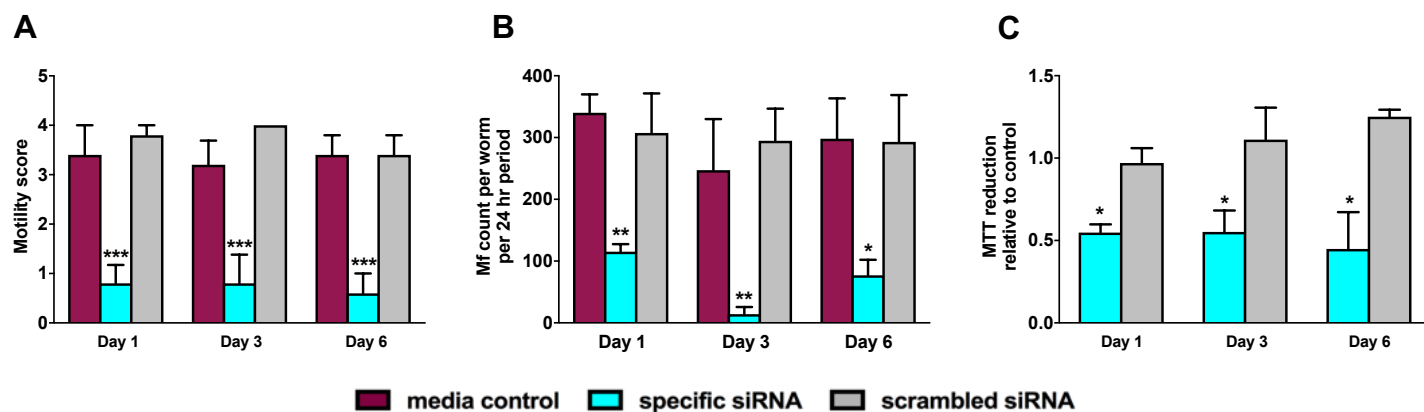


Figure 6. *Bma*-iUGT expression knockdown decreases worm motility, microfilaria release (Mf), and metabolism.

Bma-iUGT knockdown in female *B. malayi* adult worms caused reductions in (A) motility, (B) microfilaria count per worm per 24-hr period, and (C) metabolism as measured by MTT reduction relative to the media control at timepoints 1, 3, and 6 days post-siRNA treatment. * for p values <0.05, ** for p values <0.01, and *** for p values <0.001

from the specific siRNA-treated worms compared to the scrambled siRNA-treated group ($p=.0048$, Fig 6B). The greatest reduction in Mf release occurred at day 3 and was marked by a 95.25% difference between the *Bma*-iUGT siRNA group and the scrambled control (mean number of Mf release in 24h = 14 vs 294.8, $p=.0096$).

Finally, we evaluated metabolism using a (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, which measure mitochondrial oxidoreductase activity. Decreased MTT reduction by the *Bma*-iUGT siRNA treated *B. malayi* was observed at all three timepoints (Fig 4C). The values observed were similar over the course of the experiment with day 6 post-siRNA incubation showing the greatest difference (64.2%, $p=.0243$) between the specific and scrambled siRNA groups.

Treatment with UGT inhibitors exhibits macrofilaricidal activity *in vitro*

The dramatic decrease observed in worm viability and fecundity after siRNA inhibition demonstrated that *Bma*-iUGT is an essential protein for adult *B. malayi* survival. We next sought to evaluate the effects UGT inhibitors had on adult worm survival. We tested two non-specific UGT inhibitors for activity against *B. malayi* adult worms *in vitro*. Both of these agents, sulfinpyrazone and probenecid, are FDA-approved medications that are used to treat gout [158-161]. We incubated adult worms at various concentrations and found that both drugs killed filariae *in vitro* using worm motility as a metric for viability.

For sulfinpyrazone, we observed a dose-response relationship for macrofilaricidal activity *in vitro* (Fig 7A). The most rapid decline in adult worm

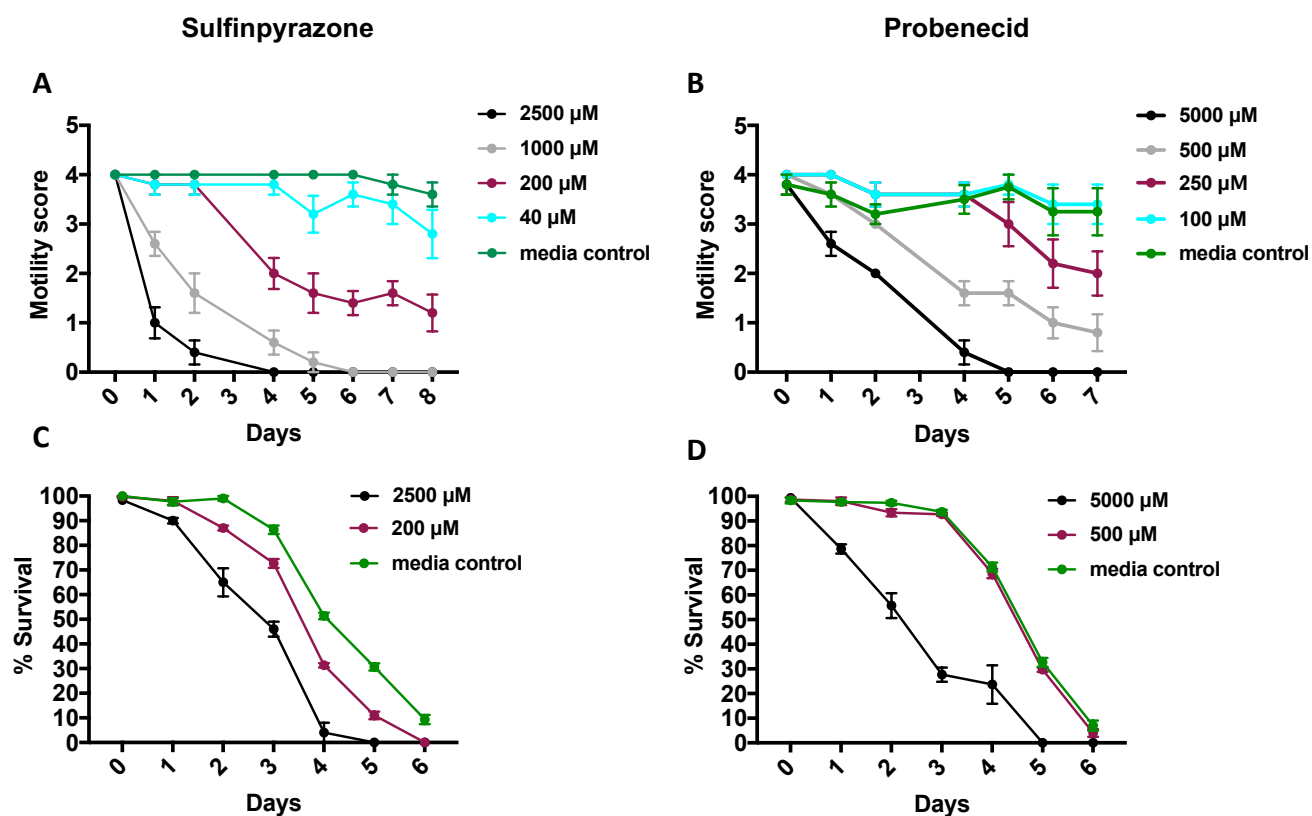


Figure 7. UGT inhibitors exhibit macrofilaricidal activity *in vitro*.

Motility scores of adult *B. malayi* adult worms after incubation with various concentrations of (A) sulfipyrazone and (B) probenecid. Percent survival of *B. malayi* microfilariae incubated with (C) sulfipyrazone and (D) probenecid.

motility occurred at 2500 μ M. At this concentration, the area under the curve (AUC) was 3.6 and significantly different than the AUC of 31.6 for the control worms incubated with media alone ($p < .0001$). Macrofilaricidal activity was also seen at 200 μ M. The AUC at this concentration was 19.7 and significantly different ($p < .0001$) than the AUC for media control. For probenecid, we also observed a dose-response curve for macrofilaricidal activity *in vitro* (Fig 7B). The greatest reduction in worm motility occurred at 5000 μ M, which had an AUC of 8.1 ($p < .0001$) compared to the media control AUC of 24.18. The lowest concentration that exhibited a significant effect on motility was 500 μ M ($p = .0004$). Though worm motility at 250 μ M was not significantly different over the course of the experiment, at day 7 there was a significant difference ($p = .0105$) between the treatment group compared to the control. While these drugs are FDA-approved, we wanted to determine whether the concentrations used were cytotoxic. Employing a lactate dehydrogenase (LDH) cytotoxicity assay with human embryonic kidney (HEK) cells, we did not detect any cytotoxicity at the concentrations that exhibited macrofilaricidal activity (Table 3).

After testing the drugs on adult filariae, we tested for microfilaricidal effect. We observed some microfilaricidal effects at the highest concentrations for both drugs (Fig 7C-D). However, no clear microfilaricidal effect was demonstrated for probenecid at 500 μ M and very little for sulfinpyrazone at 200 μ M.

Serum from filarial patients does not contain IgG and IgE antibodies specific for *Bma*-iUGT

Sulfinpyrazone		Probenecid	
Concentration	% Cytotoxicity	Concentration	% Cytotoxicity
2500 μ M	-4.14	5000 μ M	-2.49
1000 μ M	-4.46	500 μ M	-1.34
200 μ M	-5.35	250 μ M	-.67
40 μ M	-4.03	100 μ M	-2.69

Table 3. LDH Cytotoxicity Assay for the UGT Inhibitors

2 x 10⁴ HEK cells were seeded in various concentrations of sulfinpyrazone and probenecid. LDH activity was measured by absorbance at 490 nm and 680 nm and was calculated by subtracting LDH activity at 680 nm from activity at 490 nm. Percent cytotoxicity was calculated against the maximum LDH activity (lysed cells) using spontaneous LDH activity (vehicle only) as a background control.

A major obstacle for helminth vaccine development is the potential for individuals living in endemic countries to have pre-existing antigen-specific IgE and thus be at risk for developing allergic reactions when vaccinated [87]. In this study we employed a luciferase immunoprecipitation system assay (LIPS) to determine whether individuals infected with filariae developed *Bma*-iUGT-specific antibodies. Lysate containing *Bma*-iUGT-luciferase fusion protein was incubated with serum from *W. bancrofti* infected individuals (n=30) that were asymptomatic microfilaremic patients, chronic pathology (lymphedema), tropical pulmonary eosinophilia and endemic normals. Healthy normal blood bank donor sera were used as negative controls, while anti-*Bma*-iUGT peptide antibodies raised in New Zealand rabbits served as positive controls. There were no detectable levels of total IgG against The LIPS assay did not detect any *Bma*-iUGT-specific IgG (Fig 8A) or IgE (Fig 8B) in any of the serum samples from filaria patients. As expected, there was no specific IgG or IgE in serum from U.S. blood bank donors while the *Bma*-iUGT peptide IgG antibodies recognized our fusion protein. Given these results, we conclude that UGT does not elicit an antibody response during natural infection.

Adult *B. malayi* worms are able to ingest antibody

In our final experiment, we investigated whether adult filarial worms are able to ingest IgG. Previously, McGonigal et al. demonstrated that *D. immitis* is able to readily ingest fluorescein isothiocyanate (FITC)-conjugated serum [126]. We incubated 10 adult *B. malayi* female worms in Cy3-labeled antibody for 24 hrs and then looked for signal in the intestine using fluorescent microscopy. We visualized

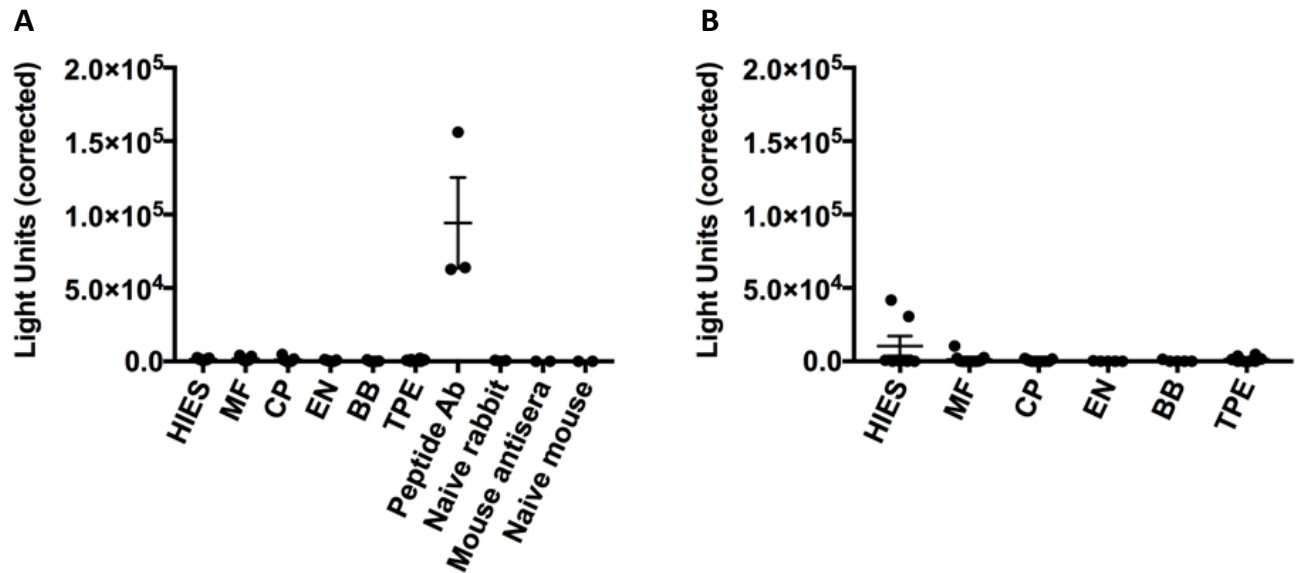


Figure 8. Serum of filaria-infected individuals does not contain detectable IgG or IgE against *Bma*-iUGT.

Filarial patient serum was incubated with a *Bma*-iUGT-luciferase fusion protein. No detectable (A) IgG and (B) IgE was measured using the LIPS assay. The *Bma*-iUGT peptide rabbit polyclonal antibodies served as a positive control for the detection of IgG. HIES=hyper IgE syndrome, MF=microfilaremic, CP=chronic pathology, EN=endemic normal, BB=blood bank donors, TPE=tropical pulmonary eosinophilic, Peptide Ab=*Bma*-iUGT peptide polyclonal antibodies, Mouse antisera=serum from *Litmoisoides sigmodontis* vaccinated mice.

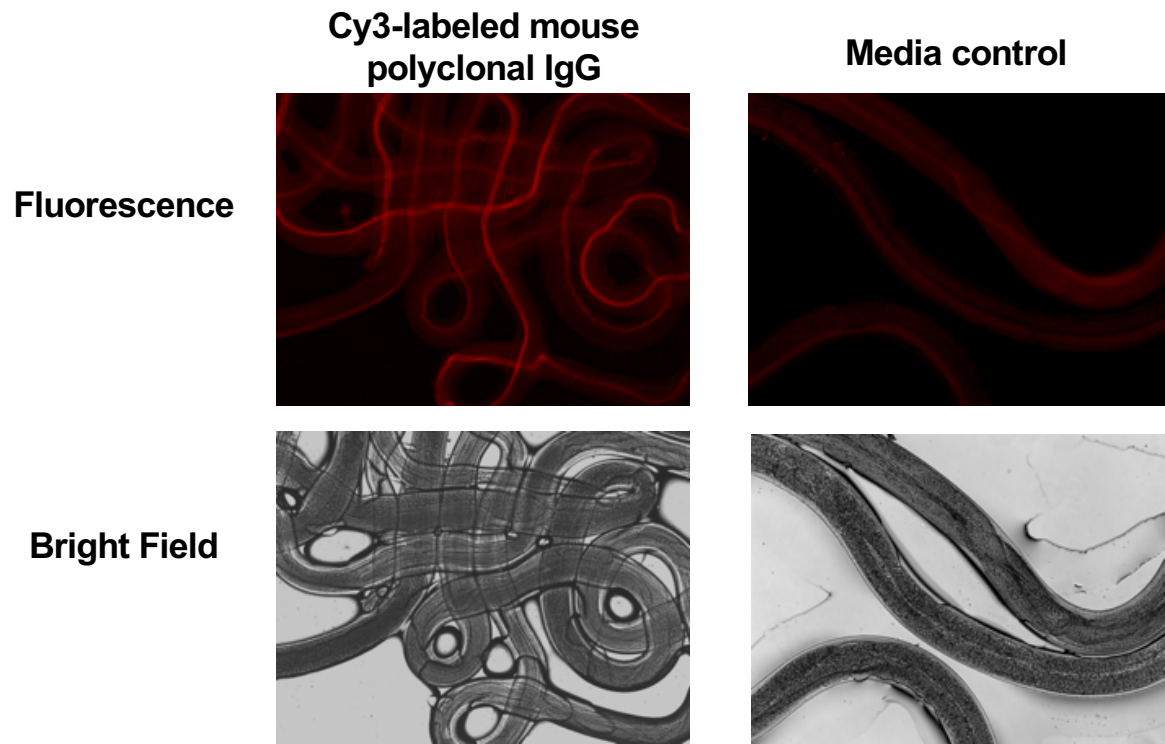


Figure 9. Adult *B. malayi* ingest mouse polyclonal IgG

Adult worms were cultured for 24 hrs with Cy3-labeled mouse polyclonal IgG or in media alone. Ingested antibody is visible in the intestinal of the adult filaria.

labeled antibody in the intestinal tract of 1 of the 10 adult filarial worms (Fig 9). On repetition of this experiment we observed uptake of fluorescence into the intestinal tract of 2 out of 10 adult filarial worms. These results demonstrate the capability of adult filariae to ingest antibody. They also suggest that adult filarial worms, at least during in vitro culture conditions, ingest macromolecules into their intestinal tract only intermittently.

DISCUSSION

UDP-glucuronosyltransferases (UGT) are enzymes important for detoxification of xenobiotics and homeostasis of endogenous molecules [162]. Specifically, these phase II enzymes increase the solubility of hydrophobic molecules by attaching sugar moieties such as glucuronic acid. Because this sugar molecule is negatively charged at physiological pH, anion efflux pumps are able to transport these molecules outside the cell [163]. In *C. elegans*, studies demonstrated that RNAi of detoxification enzymes resulted in lethality, sluggish movement, or impaired growth [164-166]. In addition, one study showed that glycosylation by phase II enzymes was important for the detoxification albendazole in *C. elegans* [167]. While UGTs in helminths have not been studied extensively, there is evidence from intestinal helminths to suggest that these enzymes play a critical role in drug resistance [168-170].

In this study, we showed that *B. malayi* intestinal UGT expression could be silenced by specific siRNA. This knockdown caused a significant reduction in worm motility, fecundity, and metabolism. We conclude that this protein is essential for

adult *B. malayi* worm survival, and that these phenotypic changes occurred due to an imbalance in endogenous molecules. Past studies in mice and rats demonstrated that UGTs were critical for protection against free radicals, which if left unchecked could mediate damage to DNA, lipid membranes, and amino acids [171-174]. However, the reduction in microfilaria release was most likely due to decreased adult worm viability because *Bma*-iUGT is not expressed in the Mf stage [155, 156]. We suspect that targeting *Bma*-iUGT *in vivo* would leave the worms not only susceptible to endogenous free radicals but also to those released by host immune cells.

Interestingly, it should be noted that the *Brugia* intestinal UGT was predicted by Interpro to be localized to the plasma membrane, which would be a novel location for this family of enzymes that are typically found in the endoplasmic reticulum. The prediction software also determined that this protein has a large extracellular domain, which potentially makes it readily accessible to drugs or antibodies. To determine if this protein is localized to the membrane, we plan to perform immunohistochemistry.

As mentioned previously, development of short-course macrofilaricidal agents would greatly enhance LF eradication efforts. Because the aim of this study was to evaluate *Bma*-iUGT as a potential drug and vaccine target, we investigated the effects of non-specific UGT inhibitors on adult filariae. We found two inhibitors that were FDA-approved to treat gout [158-161]. Both drugs, sulfinpyrazone and probenecid, exhibited macrofilaricidal activity *in vitro*. The lowest effective concentration for sulfinpyrazone was 200 μ M. A previous study investigating

sulfinpyrazone showed a maximum concentration (C_{max}) in humans of 79.9 μ M for a 400 mg dose [175]. Given that the daily maximum recommended dose for humans is 800 mg, we believe a C_{max} similar to our lowest effective sulfinpyrazone concentration may be achievable in humans [176]. Therefore, we speculate that this drug could serve as a novel therapeutic against adult filariae. Likewise, we believe the same applies to probenecid, which demonstrated robust macrofilaricidal activity *in vitro* at 500 μ M. We also observed a significant reduction in motility by day 7 at 250 μ M suggesting that this concentration may be effective at killing adult worms if given over a longer time course. In context of physiological relevance, one pharmacokinetic study showed the peak concentration in humans given a single 2 g oral dose of probenecid to be 148.6 μ g/mL (520.7 μ M) with minimal adverse events [177]. Based on our *in vitro* data, this concentration would rapidly kill adult filarial worms.

While we are interested in developing a drug that kills adult filarial worms, it could also be advantageous if it is not markedly microfilaricidal. Current antifilarial therapeutics such as diethylcarbamazine (DEC) and ivermectin (IVM) are extremely effective at clearing microfilariae. However, their use is contraindicated in areas co-endemic to loiasis and onchocerciasis because rapid killing of microfilariae in these infections can lead to severe adverse outcomes [74]. Individuals with loiasis when treated with IVM or DEC have a significantly higher risk of experiencing severe neurologic events such as encephalopathy due to rapid Mf death in the vasculature [78, 79]. Similarly, DEC can induce adverse systemic reactions such as skin lesions, fever, polyarthritis, and ocular reactions in patients

with onchocerciasis as determined by Mf load [76, 77]. Therefore, probenecid, which demonstrated macrofilaricidal and not microfilaricidal activity at 500 μ M *in vitro*, may be an attractive LF treatment candidate in these co-endemic areas.

As previously mentioned, these UGT inhibitors are FDA-approved and have been shown to be safe in humans [178]. If these drugs demonstrate macrofilaricidal activity *in vivo*, translation into human use could occur quickly.

In addition to being a potential drug target for filariasis, we postulate that *Bma*-iUGT could serve as a vaccine candidate as well. One of the challenges in helminth vaccine development is the risk that the vaccine may induce an allergic response in endemic populations. Indeed, generalized urticaria was seen in several Brazilian patients immunized against *Ancylostoma*-secreted protein 2 during hookworm vaccine trials [87]. A solution to this obstacle is to identify “hidden antigens” which are not exposed to the immune system during natural infection [113, 179]. In theory, these proteins would not elicit an IgE-mediated response, and, as postulated by Munn, these antigens may be especially vulnerable to the immune system due to a lack of evolutionary pressure to evade it [113]. There is evidence to support that the intestinal tract of nematodes contains hidden antigens. Studies have demonstrated the absence of pre-existing IgE in serum from endemic populations against hookworm worm intestinal antigens APR-1 and GST [114, 115, 119]. Furthermore, studies have shown that these antigens are protective in animal models [114, 116, 117]. There is also evidence of hidden antigens in *H. contortus* seen with the lack of an antibody response against H11, a glycosylated intestinal protein [180]. In this study, we did not detect *Bma*-iUGT-specific IgE in

the serum from filarial patients, which suggests this antigen may be safe to administer as a vaccine candidate in endemic populations.

After demonstrating that recombinant *Bma*-iUGT would not induce an allergic response in endemic populations, we investigated whether adult *B. malayi* worms could ingest antibody as there is a degree of uncertainty about whether filarial worms use their intestine to feed. Only the adult and L4 stages of filariae have a fully formed intestinal tract, while the Mf, L2, and L3 stages have an immature intestine inaccessible to nutrients [120]. Furthermore, past studies have already shown that *Brugia* worms are able to absorb nutrients such as nucleotides, amino acids, sugars, and vitamins through their cuticle, which calls into question the purpose of the intestinal tract [13]. Notwithstanding these findings, Attout et al. investigated the intestinal tract of *L. sigmodontis* and demonstrated that young adult worms (25-56 weeks post-infection) ingested red blood cells [125]. This suggests that intestinal feeding may occur but only at the early adult stage. Another study showed that *D. immitis* can ingest labeled serum [126]. With our study, we were able to show that adult *B. malayi* worms ingested Cy3-labeled IgG. This serves as clear evidence that circulating antibody can access the intestine of adult filarial worms. However, not all the worms ingested the labeled antibody, which indicates that, at least during in vitro conditions, adult worms do not feed through the intestine continuously. Future studies will work towards recombinant expression of *Bma*-iUGT in order to test for its ability to induce protective immune responses in animal models of filariasis.

On the basis of the phylogenetics analyses we conducted, we expect that *Bma*-iUGT is also essential in *W. bancrofti* and *B. timori* given the overall high homology shared between these species and *B. malayi*. Additionally, there is a high level of sequence homology (> 70%) between the *B. malayi* intestinal UGT and the orthologs found in *D. immitis* and *Loa loa* and thus the UGT orthologs in these filaria species may also serve as novel therapeutics targets. Interestingly, we did not find an ortholog of *Bma*-iUGT in *O. volvulus* and speculate this could be the result of evolutionary pressure to lose this gene.

In this study we found that *Bma*-iUGT is an essential intestinal protein in *B. malayi* adult worms that does not induce IgE antibodies in endemic populations. Importantly, we found that sulfinpyrazone and probenecid, two commercially available, FDA-approved medications in use for gout, exhibit strong macrofilaricidal activity *in vitro* at concentrations that are achievable in humans. This promising data warrants future investigation in animal models, as well as to assess whether these UGT inhibitors exhibit macrofilaricidal activity *in vitro* against other filarial species. In terms of vaccine development, future studies will focus on recombinantly expressing *Bma*-iUGT and then testing whether it is protective in animal models of filariasis. Finally, the results of this study suggest that the intestinal tract of filarial nematodes may be a rich source of essential proteins that may be able to serve as important therapeutic targets.

Chapter 4: Evaluating intestinal adhesion molecule *Bma*-LAD-2 in *Brugia malayi* adult worms as a novel drug and vaccine target

INTRODUCTION

Over 70 million people are infected worldwide with lymphatic filariasis (LF), a debilitating disease characterized by severe lymphedema, elephantiasis, and hydrocele [1]. This is caused by the parasitic nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. Currently, efforts to eliminate this disease have been spearheaded by the Global Programme to Eliminate Lymphatic Filariasis [52]. While this campaign has greatly reduced the overall prevalence of the disease, new strategies and therapeutics are needed in order to eliminate LF [73, 151, 181].

We believe that the development of new therapeutics should focus on targeting adult filariae. The current antifilarial drugs diethylcarbamazine (DEC), ivermectin (IVM), and albendazole effectively target the microfilariae (Mf), but lack robust macrofilaricidal activity [56, 68, 182]. In addition, DEC and IVM cannot be administered empirically in areas endemic for *Loa loa* or *Onchocerca volvulus* because the drugs can precipitate severe side effects by rapid killing of Mf [76-79].

Thus, a protective vaccine could greatly help efforts to eradicate LF. However, development of such a vaccine has a number of obstacles to overcome.

Because helminths elicit a robust IgE-response in infected individuals, vaccines with helminth antigens have the potential to induce allergic responses in endemic populations [87]. A possible solution to this problem is the use of “hidden antigens” in a vaccine [113]. These are proteins that are not normally exposed to the immune system during natural infection, yet which are targetable by antibodies or cellular responses after vaccination.

A potential source of such “hidden antigens” in adult helminths is the nematode intestinal tract. Indeed, there have been numerous studies demonstrating protection using intestinal antigens from hookworms and barber pole worms [114-117, 153, 154]. Furthermore, there seems to be little specific IgE in the sera of infected animal models as well as previously exposed individuals [118, 119]. This suggests that these antigens would be safe to administer in endemic areas. Given this promising data, our lab performed a proteomic analysis of the body wall, gut, and reproductive tract of *B. adult* worms [48]. We identified 106 proteins enriched in the intestine, and then selected 8 for evaluation as potential drug and therapeutic targets. The selection criteria were based on the proteins having high homology with orthologs in other filarial species and low homology to humans, a large extracellular domain potentially accessible to drugs and antibody, and a predicted function likely essential for adult filaria survival.

In this study, we identified one intestinal protein, *Bma*-LAD-2 (Bm1_39630), as a potential therapeutic target out of the 8 evaluated proteins. In adult filariae, this protein is believed to be involved in cell adhesion at the apical junction of the intestinal epithelium. By siRNA inhibition, we knocked down expression of *Bma*-

LAD-2, which resulted in decreased worm motility, metabolism, and Mf release. Using transmission electron microscopy (TEM), we observed the loss of microvilli in the intestine of worms treated with *Bma*-LAD-2 siRNA. Finally, we analyzed the antibody response in endemic populations to *Bma*-LAD-2 and found little preexisting specific IgE.

RESULTS

***Bma*-LAD-2 is phylogenetically related to orthologs found in other filarial worms**

Bma-LAD-2 has previously been shown to be a protein highly localized to the gut of adult *B. malayi* worms and to have a high predicted sequence homology ($\geq 75\%$) with other filarial orthologs [48]. For this paper, we generated a phylogenetic tree to view the level of evolutionary relatedness between the *Bma*-LAD-2 and orthologs from other filarial species. In addition, we investigated whether there was a degree of relatedness between the orthologs found in helminths such as *Ancylostoma duodenale* (hookworm) and *Haemonchus contortus* (barber pole worm) to our protein. As an outlier group for the phylogenetic tree, we included dogs, humans, and cats. These species were expected to have a significantly distant relation to *Bma*-LAD-2 given the low predicted sequence homology ($< 45\%$) between them. The tree (Fig 10) was constructed based on the likelihood estimation method for the LG model using aligned sequences by Multiple Sequence Comparison by Log-Expectation (MUSCLE). We see a close phylogenetic relation between *Bma*-LAD-2 and

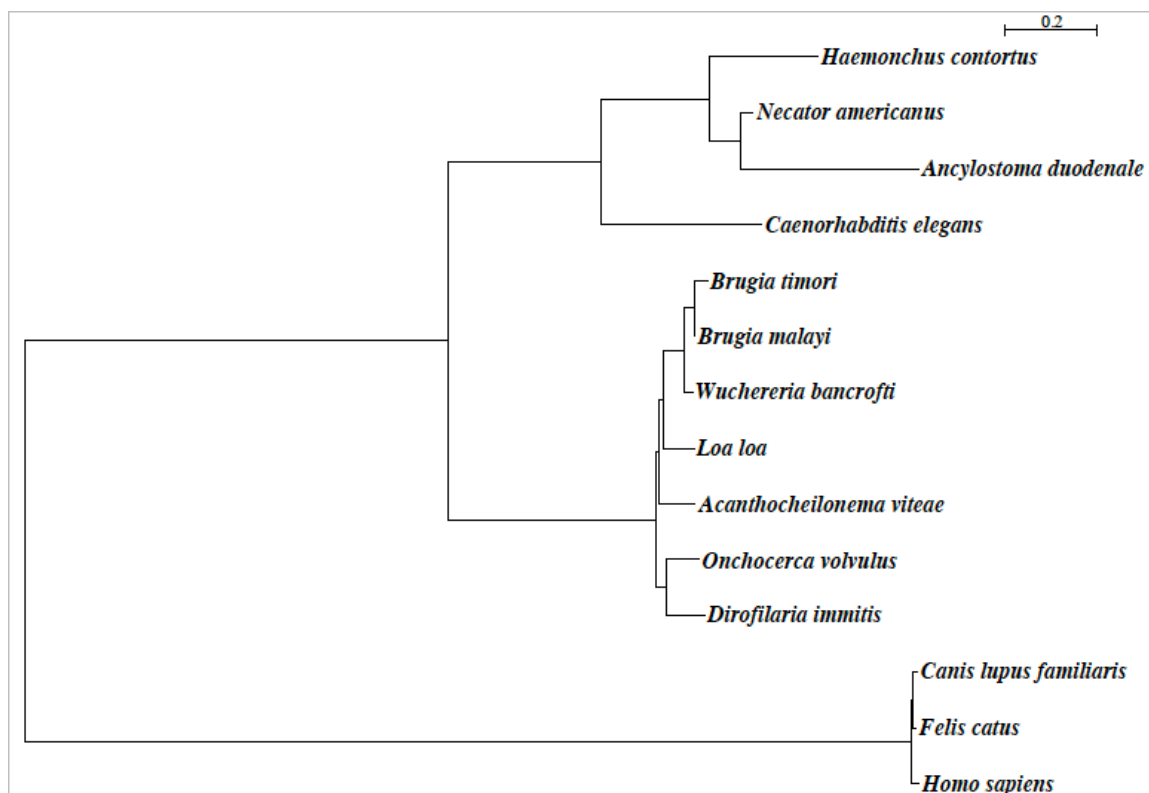


Figure 10. Phylogenetic tree of *Bma-LAD-2* and orthologs from other helminths.

The cDNA sequence of *Bma-LAD-2* has high level of relatedness to other filarial species and is evolutionarily distant from cats, dogs, and humans. Based on sequence alignment using MULTiple Sequence Comparison by Log-Expectation (MUSCLE), the phylogenetic tree was constructed by the maximum likelihood method. The phylogenetic scale indicates genetic change as measured by the number of nucleotide substitutions per site.

orthologs found in other filarial species. There was a degree of relatedness between *Bma*-LAD-2 and orthologs of intestinal helminths. Furthermore, the large phylogenetic distance to orthologs in humans, dogs, and cats suggests that targeting this protein would only effect filariae with little risk to the host.

Bma*-LAD-2 is expressed throughout the lifecycle of *B. malayi

We next investigated whether our protein was expressed in a stage-specific manner. Based on data obtained in a study evaluating the proteome of different life cycle states [156], *Bma*-LAD-2 is found to be expressed during Mf, L3, and in both genders of adult worms (Table 4). The study matched 3 unique peptides to *Bma*-LAD-2 from Mf, 1 from the L3 stage, 2 from adult females, and 1 from adult males. The similar spectra values for each sample indicates fairly consistent expression of *Bma*-LAD-2 across the larval stages.

Cy3-labeled siRNA enters the intestinal tract of *B. malayi* adult worms

Prior to siRNA knockdown, we investigated whether *Bma*-LAD-2 siRNA 1 conjugated to Cy3 could be visualized in the intestinal tract of adult filariae. We incubated the worms with labeled siRNA for 24 hrs and then observed them under an epifluorescent microscope. The Cy3-labeled siRNA (Fig 11A-B) was distinctly seen in the intestinal tract of the treated worms. As expected, minimal signal was visualized in the intestine of worms treated with only culture media (Fig 11C-D). We therefore concluded that siRNA targeting *Bma-lad-2* transcript could enter the intestinal tract.

	Adult Male	Adult Female	L3 Larvae	MF	Immature MF
<i>Bma</i> -iUGT protein	X	X	X	X	X

Table 4. Stage specific analysis of *Bma*-LAD-2 in *B. malayi*

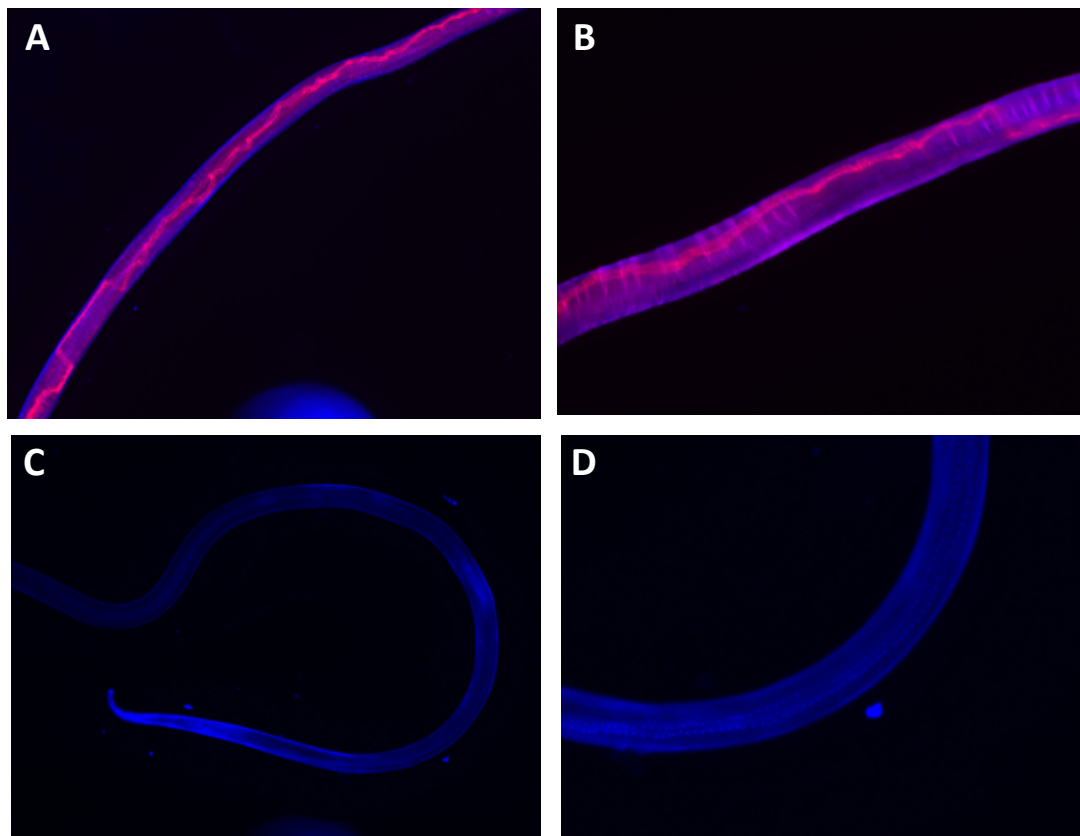


Figure 11. Cy3-labeled *Bma*-LAD-2 siRNA entry into the intestinal tract of female *B. malayi* adult worms.

B. malayi adult female worms soaked in Cy3-labeled siRNA (red) for 24 hrs and visualized at magnifications of (A) 100x and (B) 200x. As a negative control, worms were cultured in only media for 24 hrs and visualized at magnifications of (C) 100x and (D) 200x. Worms were counterstained with DAPI (blue).

Bma-LAD-2 siRNA treatment results in reduced transcript and protein expression

After observation of *Bma*-LAD-2 siRNA entry into the intestine, gene and protein expression was evaluated by quantitative reverse transcription PCR (RT-qPCR) and Western blot respectively. cDNA was generated using mRNA isolated from filariae cultured in media alone, *Bma*-LAD-2 siRNA, and scrambled siRNA for 1 day and 6 days post-siRNA incubation. Using Taqman primers, we quantified *B. malayi lad-2* gene expression normalized to *Bma-gapdh*. We observed a 70.42% decrease in *Bma-lad-2* transcript levels (Fig 12A) in worms treated with target specific siRNA (mean=.2662) compared to the scrambled siRNA-treated filariae (mean=.9) relative to the media control group at 1-day post-siRNA incubation. *Bma*-LAD-2 protein expression was visualized by Western blot (Fig 12B) in worms treated with specific or scrambled siRNA for 24 hrs and then cultured in media for an additional 24 hrs. We probed for the protein using polyclonal antibodies raised in New Zealand rabbits against recombinant *Bma*-LAD-2. A dramatic decrease in *Bma*-LAD-2 expression was observed in the specific siRNA-treated worms compared to the controls.

Reduced worm viability and fecundity in *Bma*-LAD-2 siRNA-treated adult filariae

We evaluated the effects of *Bma*-LAD-2 knockdown on worm motility, Mf release, and (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. We scored worm motility on the following scale: 0 = no movement, 1 =

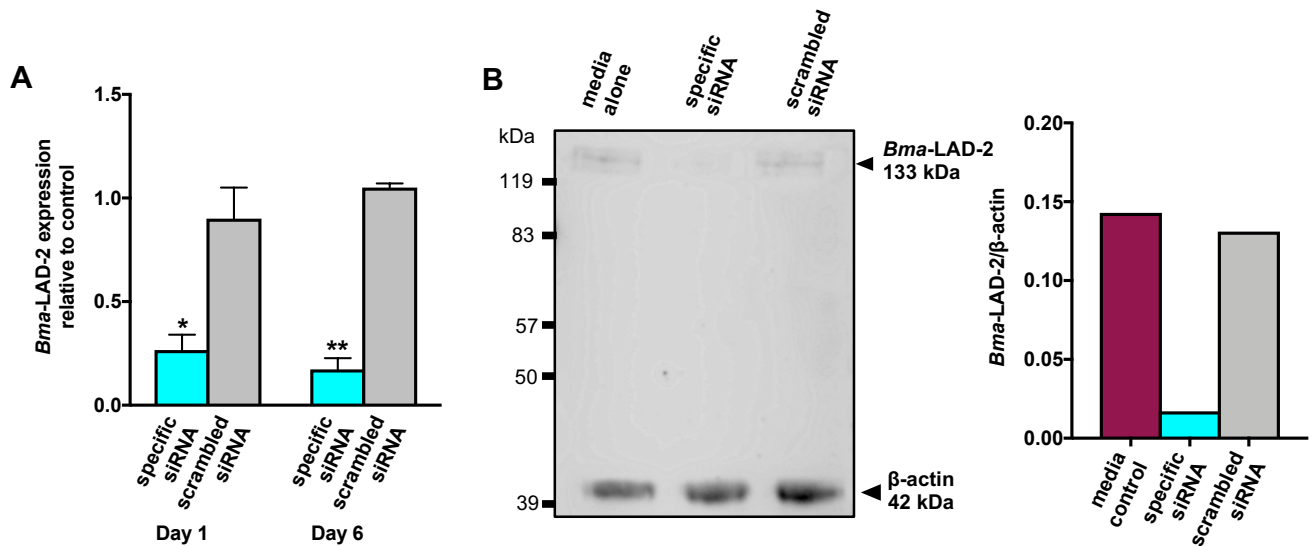


Figure 12. Treatment with *Bma*-LAD-2 siRNA reduces target transcript and protein levels in adult *B. malayi* worms.

Adult filaria were cultured in *Bma*-LAD-2 siRNA, scrambled siRNA, or media alone. (A) *Bma-lad-2* expression was reduced for the specific siRNA-treated groups compared to the scrambled controls as measured by qPCR. Gene expression was normalized to *Bma-gapdh*. (B) *Bma*-LAD-2 levels were assessed 24 hrs post-siRNA incubation by Western blot using anti-*Bma*-LAD-2 antibodies. * for p values <0.05, ** for p values <0.01, and *** for p values <0.001

twitching, 2 = severe reduction in movement, 3 = moderate reduction in movement, and 4 = active movement. At day 1 post-siRNA incubation, worm motility (Fig 13A) was significantly reduced ($p<.0001$) in worms soaked in *Bma*-LAD-2 siRNA (mean=.8) compared to the scrambled control group (mean=4). By day 6 post-incubation, we observed a 100% reduction in motility for the specific siRNA-treated group (mean=0) compared to the scrambled siRNA group (mean=4). We next evaluated Mf release per adult worm per 24 hr period (Fig 13B) for each group at timepoints 1 and 6 days post-siRNA incubation. The only significant difference in Mf release between the treatment and control groups occurred at day 6. We observed a 93.43% reduction in Mf release from *Bma*-LAD-2 siRNA soaked adult filariae. Finally, 2 randomly selected adult worms from each group for each timepoint were used for the MTT reduction assay (Fig 13C). At day 1 post-siRNA treatment, there was a significant reduction ($p=.0139$) in MTT reduction by *B. malayi* treated with target siRNA (mean=.435) compared to worms treated with scrambled siRNA (mean=.9303) relative to the media control group. By day 6, we observed an 83.25% decrease in MTT reduction from the *Bma*-LAD-2 siRNA group. Given these results, we conclude that *Bma*-LAD-2 is an essential protein for *B. malayi* adult worm survival *in vitro*.

Transmission electron microscopy (TEM) reveals ablation of microvilli in the intestinal tract of *Bma*-LAD-2 siRNA-treated adult filariae

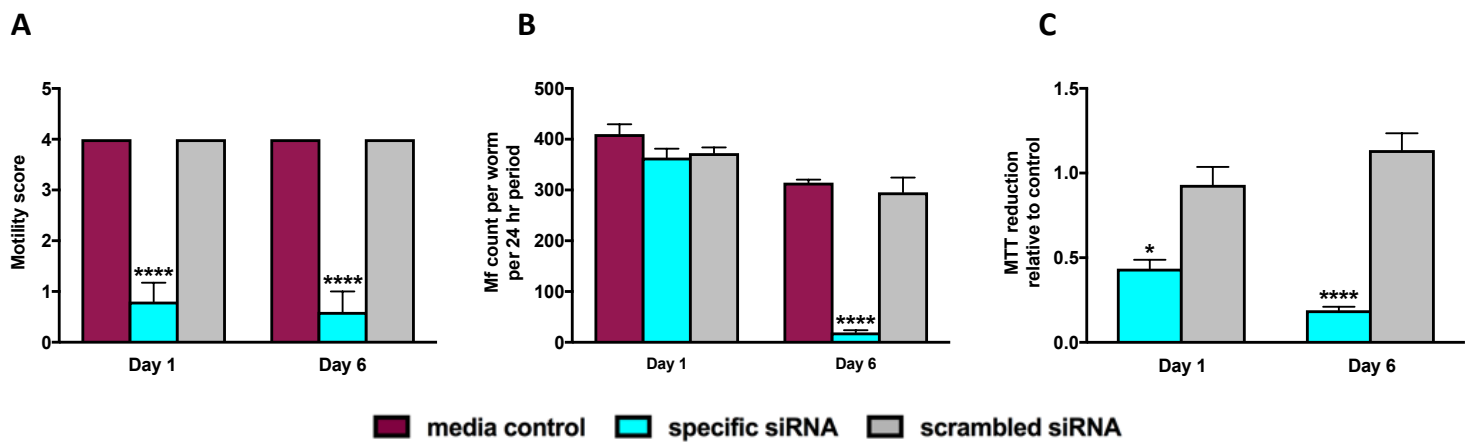


Figure 13. *Bma*-LAD-2 knockdown results in decreased worm motility, microfilaria release (Mf), and metabolism.

Reducing *Bma*-LAD-2 expression in female *B. malayi* adult worms resulted in decreased (A) motility, (B) microfilaria count per worm per 24-hr period, and (C) metabolism as measured by MTT reduction at timepoints 1 and 6 days post-siRNA treatment. * for p values <0.05, ** for p values <0.01, and *** for p values <0.001

Bma-LAD-2 was predicted to be an adhesion protein located at the apical junction of the intestinal tract. Therefore, we evaluated the structure of the adult filaria intestinal tract after treatment with *Bma*-LAD-2 siRNA. By TEM, we visualized microvilli lining the epithelium of the intestinal tract (Fig 14) of normal *B. malayi* adults. Upon treatment with *Bma*-LAD-2 siRNA, there was a robust change in the ultrastructure of the intestine (Fig 15). We observed near complete ablation of the microvilli in the intestine of the treated worms. In addition, many of the apical junctions in the intestinal tract of treated worms appeared shortened suggesting the loss of adhesion molecules. Finally, we noticed that many of the mitochondria in the treated worms had unraveled cristae.

No detectable *Bma*-LAD-2-specific IgG or IgE in serum from filarial patients

A clinical study evaluating the hookworm vaccine, *Necator americanus* Ancylostoma-secreted protein 2, demonstrated that helminth antigens could elicit an allergic response in previously infected individuals [87]. Considering this result, we investigated whether serum from filaria-infected individuals contains IgE that recognizes *Bma*-LAD-2. The luciferase immunoprecipitation system assay was used to detect antibody levels in the serum samples. In this system, a *Bma*-LAD-2-luciferase fusion protein was incubated with serum in a 96-well filter plate. Antibody was captured using protein A/G beads for IgG and anti-IgE beads for IgE. Applying a vacuum to the plate left only captured antigen-antibody complexes in the wells. We detected the presence of the fusion protein by adding coelenterazine

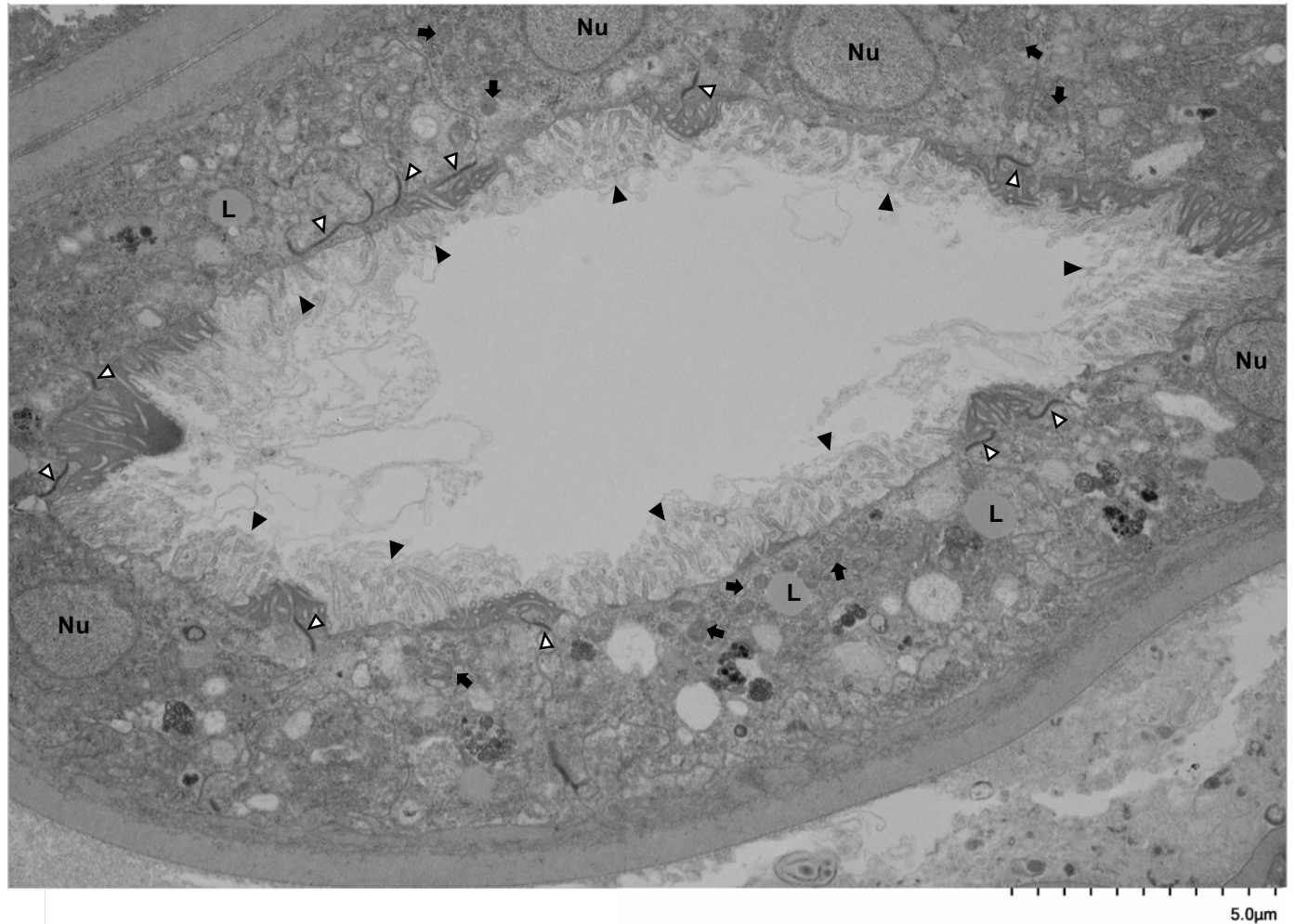


Figure 14. Intestinal tract of a *B. malayi* adult worm.

Image was captured by transmission electron microscopy (TEM) at 4,000x. Adult filaria were cultured in media alone for 72 hrs. Microvilli (black arrowhead) line the apical surface of the intestinal epithelium. Other structures visible are apical junctions (white arrowhead), nuclei (Nu), lipid droplets (L), and mitochondria (black arrow).



Figure 15. Intestinal tract of a *B. malayi* adult worm treated with *Bma*-LAD-2 siRNA.

Image was captured by transmission electron microscopy (TEM) at 4,000x. Adult filaria were incubated with *Bma*-LAD-2 siRNA for 24 hrs and then cultured in media alone for an additional 48 hrs. Microvilli (black arrowhead) are largely absent from the apical surface of the intestinal epithelium. There are some vestigial microvilli have been invaginated by the surrounding epithelium. Other structures visible are apical junctions (white arrowhead), nuclei (Nu), and lipid droplets (L). Many mitochondria (black arrow) appear to have unraveled cristae.

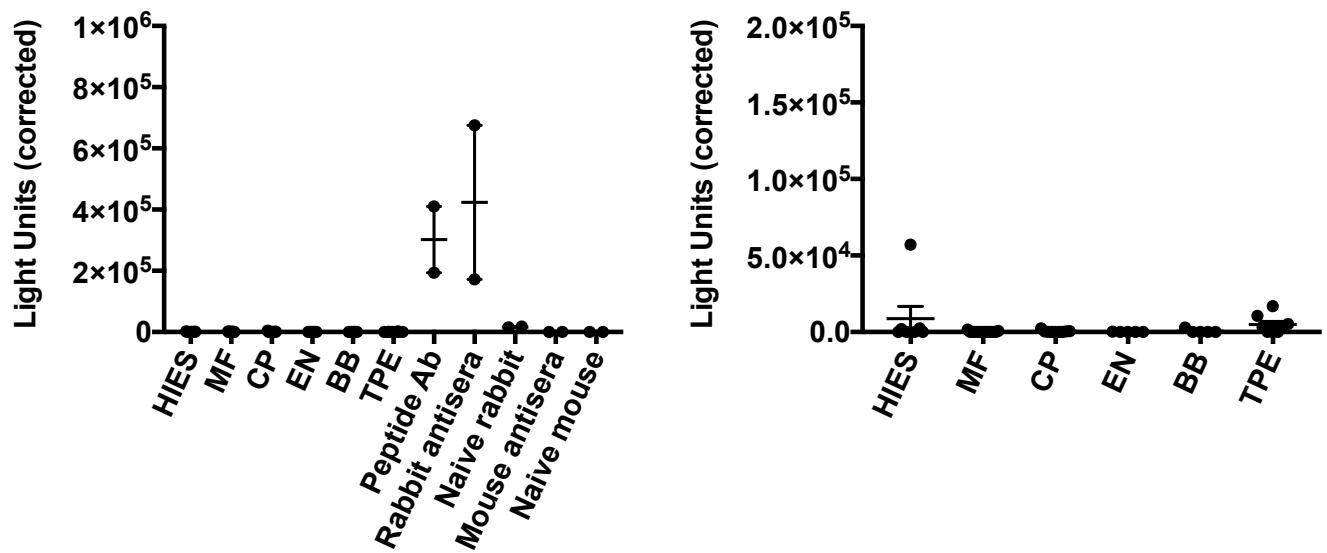


Figure 16. Filarial patient serum does not contain detectable IgG or IgE against *Bma*-LAD-2.

Serum from filaria-infected individuals was incubated with a *Bma*-LAD-2-luciferase fusion protein. There was no detectable (A) IgG and (B) IgE in the patient serum as measured by the LIPS assay. As a positive control for IgG binding, *Bma*-LAD-2 rabbit polyclonal antibodies recognized the fusion protein. HIES=hyper IgE syndrome, MF=microfilaremic, CP=chronic pathology, EN=endemic normal, BB=blood bank donors, TPE=tropical pulmonary eosinophilic, Peptide Ab=*Bma*-LAD-2 rabbit polyclonal antibodies, Mouse antisera=serum from *Litomosoides sigmodontis* vaccinated mice.

substrate and then measuring the light units generated. We found that filarial patient serum had no pre-existing IgG (Fig 16A) or IgE (Fig 16B) against *Bma*-LAD-2 fusion protein. As a positive control, we used affinity-purified polyclonal antibodies raised in rabbits immunized with recombinant *Bma*-LAD-2 as well as the rabbit anti-sera. As expected, there was recognition by the polyclonal antibodies and anti-sera against the fusion protein. This indicated that our fusion protein exhibited the proper conformation and thus the absence of signal in filarial patient samples was due to absence of *Bma*-LAD-2-specific IgG or IgE. Thus, we believe that this antigen would have a low likelihood of inducing allergic reactions if administered as a vaccine to endemic populations.

Other intestinal proteins were not essential for *B. malayi* adult worm survival

In this study, we also attempted to evaluate whether 7 other intestinal proteins were essential for adult *B. malayi* survival (Table 4). The proteins selected for investigation were annotated as adhesion molecules, proteases, protease inhibitors, or involved in glycosylation based on work from previous studies [48, 156]. We were able to successfully knockdown gene expression for 3 of the target proteins. This limited success with siRNA inhibition was not entirely unexpected given the reported variability and difficulty of performing RNA interference in parasitic nematodes [137, 183]. Of note, unlike knockdown of *Bma*-LAD-2, successful siRNA inhibition of *Bma*-Serpine (a protease inhibitor) and *Bma*-Reprolysin (a protease) did not cause any appreciable phenotypic changes in adult *B. malayi* worms. siRNA inhibition of *Bma*-ShTK, also a predicted protease,

Gene Target	Pub locus	Function	Motility	Mf Release	Metabolism	Transcript knockdown
<i>Bma-fukutin</i>	Bm1_44655	Glycosylation	No change	No change	No change	No
<i>Bma-serpin</i>	Bm1_09775	Protease inhibitor	No change	No change	No change	Yes
<i>Bma-shtk</i>	Bm1_00205	Protease	Minimal	No change	Mild	Yes
<i>Bma-reprolysin</i>	Bm1_53050	Protease	No change	No change	No change	Yes
<i>Bma-peptidase</i>	Bm1_38300	Protease	No change	No change	No change	No
<i>Bma-egf-like-02820</i>	Bm1_02820	Adhesion	No change	No change	No change	No
<i>Bma-egf-like-48010</i>	Bm1_48010	Adhesion	No change	No change	No change	No

Table 5. siRNA experiments targeting other intestinal proteins in *B. malayi* adult worms.

Target specific siRNA treatment successfully reduced transcript levels for *Bm-serpin*, *Bm-shtk*, and *Bm-reprolysin*. No significant changes in motility, Mf release, and metabolism were observed in the adult filaria.

caused only minimal decreases in adult worm motility and metabolism.

DISCUSSION

Bma-LAD-2 is an immunoglobulin (Ig) intermediate-set (I-set) domain containing protein and, therefore, belongs to the functionally diverse Ig superfamily (IgSF). The Ig domain is the basic structural unit of the superfamily and consists of two sandwiched antiparallel beta sheets [184]. Proteins in the IgSF are classified based on the structure of their Ig domain and given a set designation of variable (V), constant 1 (C1), constant 2 (C2), or intermediate (I) [185]. Ig I-set domains are similar to V-set domains but have a shorter distance between the invariant cysteine residues. InterPro analysis predicts that *Bma*-LAD-2 has 6 Ig domains spanning from amino acid position 24 to 602.

Based on homology to its ortholog in *C. elegans* (LAD-2, L1 adhesion), *Bma*-LAD-2 is predicted to be an L1 cell adhesion molecule (L1CAM). L1CAMs are single transmembrane proteins that can participate in homophilic and heterophilic interactions [186]. The cytoplasmic tail of L1CAMs has multiple consensus-binding sites which allow for interaction with various cytoskeleton linkers proteins such as ankyrin, spectrin, and ERM [187, 188]. Furthermore, the cytoplasmic tail of L1CAMs has phosphorylation sites indicating a possible role in signal transduction [186, 189, 190].

Interestingly in *C. elegans*, LAD-2 is critical in guiding axon migration and does not appear to be critical for the establishment or maintenance of the intestinal epithelium [191, 192]. However, LAD-1, another L1CAM, has been shown to co-localize with apical junction molecules. In nematodes, cell adhesion molecules

(CAMs) assemble to form two major types of apical junctions: the cadherin catenin complex (CCC) and the DLG-1/AJM-1 complex (DAC) [187, 192]. In *C. elegans*, it has been shown that the CCC is not essential for cell adhesion [193]. This is surprising given the critical role of cadherins in cellular adhesion for most other eukaryotes. Researchers have suggested that LAD-1 may act as a redundant adhesion system thereby mitigating the loss of the CCC. Indeed, embryos expressing dominant-negative LAD-1 have altered cell morphology and position indicating a role in cellular adhesion [194-196].

In addition, it has been suggested that LAD-1 interacts with the DAC, located basal to the CCC [189, 190, 192, 197]. This complex is characterized by the presence of two membrane proteins, a *Drosophila* discs large homologue (DLG-1) and apical junction molecule (AJM-1). In *C. elegans*, it has been shown that DLG-1, a membrane-associated guanylate kinase, interacts with AJM-1, a coiled-coiled protein, to maintain localization of junction molecules, act as paracellular gates, and regulate cell proliferation [192, 198]. In *C. elegans*, RNAi of DLG-1 has been shown to be embryonically lethal while mutagenesis of *ajm-1* leads to arrested development at the 2-3-fold stage of elongation [198, 199]. These molecules are thought to co-localize to the membrane by interactions with a transmembrane protein. One such protein thought to be membrane anchor for the DAC is LAD-1. However, studies have failed to demonstrate the existence of this interaction though loss of the DAC reportedly disturbs the junctional localization of phosphorylated LAD-1 [197, 200].

In this study, we observed a significant decrease in worm motility, metabolism, and microfilaria release when *Bma*-LAD-2 expression was knocked down. While it is possible that reduction of *Bma*-LAD-2 had a direct effect on these evaluated outcomes, it remains unlikely. We hypothesize that this phenotype was a secondary effect brought on by the dramatic changes in the intestinal ultrastructure visualized by TEM. Evidence indicates that L1CAMs play an integral role in the maintenance and formation of apical junctions in nematodes. Therefore, it is likely that the reduction in *Bma*-LAD-2 signaled a loss of cell-to-cell contact in the epithelial cells. Studies have shown that such a loss of L1CAM signal can arrest cell proliferation and potentially induce apoptosis [201, 202]. This would explain the ablation of microvilli in the intestine of target specific siRNA-treated worms as well as the unraveling of the mitochondrial cristae. In addition, the loss of adhesion molecules may have hindered the ability of the apical junction to prevent diffusion of the internal hemolymph into the intestinal lumen. We plan to address this possible mechanism by performing osmolarity analysis of the lumen in future studies.

The observed structural change in the intestinal epithelium could only result in a decrease in worm viability and fecundity if the intestine served an essential role in adult filaria survival. This has been a point of contention with some believing the structure is somewhat expendable. Studies have shown that *Brugia* worms can absorb some nutrients through their cuticle, casting doubt on whether these filariae need an intestine for digestion [13, 44]. However, a previous study of *Litomoisoides sigmoiditis*, a filarial worm in cotton rats, showed the presence of red blood cells in

the filarial gut which implied that adult filariae actively feed [125]. Another study demonstrated that heartworms are able to ingest serum [126]. In addition, the proteomic analysis of different filarial tissue structures performed by our lab revealed that the filarial intestine is enriched in transporters, drug metabolizing enzymes, proteases, protease inhibitors, and adhesion molecules [48]. These findings suggest that the gut is used for not only nutrient digestion and uptake but also waste metabolism and disposal; functions essential in any living organism.

The loss of *Bma*-LAD-2 results in a different phenotype than what has been observed when the ortholog of this protein is knocked out in *C. elegans*. This could be due to a number of reasons. *C. elegans* constantly use their intestine for digestion and waste disposal, emptying their intestinal contents every 45 seconds [44, 120, 203]. Additionally, *C. elegans* are unable to absorb nutrients across their thick cuticle, which leaves the intestine as the only means of nutrient absorption. Evolutionarily, it is reasonable that the systems in the intestine of *C. elegans* are redundant as failure in one could result in worm death. Indeed, we see evidence of this redundancy by the fact that knocking out the CCC does not dramatically disrupt cell adhesion in *C. elegans* [193]. In contrast, intestinal feeding by adult filariae may be more inconsistent as the parasites can absorb at least some nutrients through their cuticle [44, 120]. It is quite possible that helminths only use their intestine to digest essential proteins and macromolecules too large to be absorbed by the cuticle. Consequently, there may have been less evolutionary pressure to develop redundant systems in the intestine. Finally, *C. elegans* is a free-living nematode, and therefore, has different digestive requirements than a

parasitic nematode such as *Brugia*. This difference is no more apparent than with the number of protein-coding genes. *C. elegans* has 19,762 protein-coded genes compared to the predicted ~11,500 of *B. malayi* [156, 204].

When developing a helminth vaccine, there is a risk that the antigen could induce an allergic reaction in individuals previously exposed to filariae [119]. This has been a major impediment to the development of effective helminth vaccines. There is evidence to suggest that helminth intestinal proteins act as “hidden antigens,” which are proteins not exposed to the immune system during natural infection and thus would not elicit an IgE-response [113-115, 119, 180]. Furthermore, because these proteins are hidden from the immune system, there is little evolutionary pressure to develop mechanisms that enable these proteins to evade the immune system. This may leave these proteins vulnerable to attack by the host defenses [113]. When evaluating *Bma*-LAD-2 as a potential vaccine, the antigen does not appear to elicit an IgE-response in endemic populations. This suggests that this antigen may be safe to use as a vaccine.

In this study, we also evaluated 7 other *Brugia* intestinal proteins. Successful transcript knockdown was achieved for 3 of the targets. We attribute this failure rate to the difficulty in performing RNA interference in helminths [137, 183]. None of the other proteins that were successfully knocked down (*Bma*-Serpine, *Bma*-ShTK and *Bma*-Reprolysin) resulted in substantial changes to worm viability or fecundity. It is expected that not all intestinal proteins are essential for adult filaria survival. Interestingly, inhibiting expression of *Bma*-Reprolysin, a putative protease, did not result in a noticeable phenotype. This may be due to the

fact that there are multiple proteases present in the gut and that knockdown of more than one is necessary to affect worm survival.

Finally, we suspect that any therapeutics developed against *Bma*-LAD-2 would be effective in *W. bancrofti* and *B. timori* due to high overall relatedness between the filaria species. In addition, *Bma*-LAD-2 shares a high level of homology ($\geq 75\%$) with other filarial species. Therefore, a therapeutic or vaccine developed against this protein may be protective against other filarial infections.

In this study, we demonstrated knockdown of *Bma*-LAD-2 expression in adult *B. malayi* by siRNA inhibition. This resulted in an ablation of microvilli, shortened tight junctions, and unraveling of the mitochondrial cristae as visualized by TEM. We believe that these structural changes in the intestinal epithelium led to decreased worm motility, metabolism, and Mf release in worms treated with *Bma*-LAD-2 siRNA. Therefore, we conclude that *Bma*-LAD-2 is an essential protein for adult worm survival. The lack of *Bma*-LAD-2-specific IgE suggests that this antigen would be a safe vaccine to administer in endemic areas. Therefore, in future studies we plan to evaluate recombinant *Bma*-LAD-2 as a vaccine candidate in animal models.

Chapter 5: Discussion

MAJOR FINDINGS

We postulated that essential intestinal proteins in adult *B. malayi* worms could serve as novel therapeutic targets. To address this hypothesis, we developed 2 specific aims: (1) identify essential intestinal protein by siRNA knockdown and (2) determine whether filarial patient serum contains reactive IgE antibodies against these essential intestinal proteins. With regard to the first specific aim, we identified *Bma*-iUGT and *Bma*-LAD-2 as essential intestinal proteins in adult *Brugia* worms. Focusing on *Bma*-iUGT, we then selected 2 FDA-approved commercially available UGT inhibitors, sulfinpyrazone and probenecid, which, upon testing, demonstrated macrofilaricidal activity *in vitro* at physiologically relevant concentrations. This is an important finding because these drugs are ready for immediate use in humans if proven effective *in vivo*. Furthermore, probenecid exhibited a lack of microfilaricidal activity at the highest concentration tested, 500 μ M. This result would indicate a possible use in areas endemic for onchocerciasis and loiasis, where sudden death of Mf is of concern. Another interesting finding was the loss of microvilli, shortening of tight junctions, and unraveling of the mitochondrial cristae in the intestine of worms treated with *Bma*-LAD-2 siRNA. This suggested that the protein was critical for structural maintenance of the intestinal epithelium.

For the second aim, we employed the LIPS assay to evaluate the antibody titers in filarial patient serum against the 2 aforementioned proteins. There is a risk that a helminth antigen, which induces an IgE response [87], could cause an allergic reaction in individuals previously exposed to filariae. We did not detect the presence of specific IgE to either protein in the sera of these patients. This would indicate that these antigens may be safe to use as vaccine antigens. Furthermore, it strengthens the argument that the helminth intestine is a source of “hidden antigens.”

Limitations of the study

Despite the novel findings of this study, there are some limitations that must be addressed. First, we cultured the adult filarial worms *in vitro*. Studies have shown that there is differential gene expression when *B. malayi* adults are cultured *in vitro* [205]. It is possible that we are targeting pathways that are upregulated and needed for filaria survival outside a host. Another limitation is with the UGT inhibitor experiments. Even though they exhibited macrofilaricidal activity *in vitro*, it does not mean the same would occur *in vivo*. Indeed, there are examples of drugs that are active against adult filaria *in vitro* but lack activity in animal models [206]. This could be due to the metabolism and pharmacokinetics of the host organism. However, we believe that there is enough data to suggest that the UGT inhibitors, sulfinpyrazone and probenecid, would maintain their activity in humans as well as animal models [158-161, 170, 175-177]. In addition, when considering *Bma*-iUGT as a potential vaccine antigen, there is a possibility that the protein is expressed within the smooth endoplasmic reticulum. This would make the protein less

accessible to antibodies, however, it has been demonstrated that antibodies can be internalize and target cytosolic proteins in filariae [207, 208]. If the protein is expressed on the outer membrane, then this would truly be a novel finding for this class of enzyme [174, 209, 210]. This study demonstrated that certain intestinal proteins such as *Bma*-iUGT and *Bma*-LAD-2 are essential for adult *Brugia* survival. However, it is important to note that this does not prove that helminth feeding through the intestine is necessary. One might argue that the loss of the microvilli implies that intestinal digestion is an essential function in adult worms. However, it is possible that remodeling of the intestinal epithelium hindered functions such as waste removal or barrier maintenance, which are arguably necessary for filaria survival.

DO WE NEED NEW DRUGS TO TREAT LYMPHATIC FILARIASIS?

An argument can be made that the current antifilarials will be sufficient for eliminating LF. Thomsen et al. demonstrated long suppression of microfilaremia and possible macrofilaricidal effect of administering triple therapy (IDA; IVM, DEC, albendazole) in the endemic populations [72]. However, the study failed to address how the IDA therapy would be implemented in areas co-endemic for *O. volvulus* and *Loa loa*. Though a measured approach to IDA treatment of individuals in areas endemic to onchocerciasis has been suggested [211]. This strategy would involve pre-treating individuals with IVM to lower Mf numbers prior to administration of IDA. However, this strategy cannot be safely implemented in *Loa loa* co-endemic areas. A proposed treatment strategy for these areas is screening out at-risk individuals (>30,000 Mf/mL of blood) prior to MDA [212]. There have been two studies (2015

and 2017) in Cameroon evaluating this test and not treat (TaNT) approach [212, 213]. Of the 37,500 individuals screened in these studies, 36,600 were treated with IVM with no recorded severe adverse events. While the studies were a success, the financial and manpower cost for implementing this treatment approach is still being calculated, but it is expected to be prohibitive based on a previous pilot TaNT study in 1999 [212]. Therefore, employing such strategies may place an even heavier strain on African countries still trying to catch up with the goal objectives of the GPELF [1]. As recently as 2014, there were 20 countries that had not completed mapping the geographic distribution of LF in their borders or implemented the MDA [73].

In addition, there is evidence to suggest that filarial worms are acquiring resistance to current antifilarials. Osei-Atweneboana et al. investigated an *O. volvulus*-infected population that had responded well to initial IVM treatment with reductions in Mf of 99.3% to 100% [214]. The group reported that 25% of the microfilaridermic patients (n=268) were now responding poorly to IVM treatments administered during the study [82]. In these individuals, Mf recovery rate after a year post-treatment was between 103.8% and 115.8%. Furthermore, adult worms isolated were shown to have many live Mf *in utero*. The authors believed that acquired IVM resistance after multiple doses was the reason behind this poor response in certain test participants. This resistance could be the result of genotypic selection. One study showed that IVM treatment resulted in a shift in allele frequency and reduced polymorphism for an ATP-binding cassette (ABC) transporter (OvABC-3) in *O. volvulus* [215]. Efflux of xenobiotics by ABC

transporters has been implicated as one of potential mechanisms of drug resistance in helminths [216]. While allelic selection on this transporter may not impart IVM resistance, it does provide evidence that gene selection does occur in filariae after antifilarial treatment.

The threat of drug resistance is more apparent with helminth infections of livestock [217]. Commercial livestock are prophylactically treated with anthelmintics, which has been the industry standard since the drugs were first discovered. Consequently, there has been a rise in the prevalence (>50% in some areas) of anthelmintic resistance (AR) in sheep, goats, and horses [217, 218]. The prevalence of AR is so widespread in South Africa and Paraguay that farmers have stopped raising sheep [219, 220]. AR has been attributed to various factors such as high treatment frequency, single-drug regimens, and 100% treatment coverage. Thus, mass anthelmintic treatment of commercial livestock mirrors in many ways the deworming efforts by countries around the world. We should therefore take the necessary precautions to prevent AR from becoming prevalent with human helminth infections. One precaution would be to continue the development and identification of new anthelmintics. To this aim, our study reinforces the concept that repurposing FDA-approved may be an effective tool in the effort to eliminate LF. Given the benefits for succeeding in such an endeavor, it is only practical to pursue all possible routes to achieve that goal.

A rapid solution to develop macrofilaridal agents would be to identify drugs that kill adult filarial worms from amongst agents that are already FDA-approved and commercially available. The safety and pharmacokinetics of these drugs are

already known, mitigating the risk involved in drug discovery. Indeed, there has been an effort to repurpose drugs to target *Wolbachia*, the bacterial endosymbiont essential for adult filaria viability and fecundity [221]. One study screened 2664 FDA-approved drugs and found 121 had activity against *Wolbachia* [206]. Of these active drugs, 69 were found to be available in oral form. Other drug repurposing studies have focused on finding novel therapeutics that act directly on the Mf and adult filaria. Efforts have been made to investigate tyrosine kinase inhibitors as possible treatment options [207, 208]. These drugs block the phosphorylation of c-abl, a cytoplasmic tyrosine kinase involved in cell proliferation and survival. O'Connell et al. showed that a single dose of imatinib, an approved tyrosine kinase inhibitor, resulted in 100% Mf death after 4 days at 50 μ M and 90% adult worm death after 4 days at 100 μ M [207].

FILARIAL INTESTINAL PROTEINS AS “HIDDEN ANTIGENS”?

In this study, we identified 2 intestinal proteins, *Bma*-iUGT and *Bma*-LAD-2, as antigens to which endemic populations lack specific IgE. This reinforces the concept that filarial intestinal proteins are not exposed to the immune system and therefore act as “hidden antigens.” Though, it must be emphasized that not all intestinal proteins are expected to be hidden. Our lab's proteomic study of the body wall, intestine, and reproductive tract of *B. malayi* adult worms revealed 3,173 proteins in the intestinal tract [48]. Of these, 3,173 proteins, only a small fraction (396) were specific to the intestine. A significant portion (2,135) of the proteins found in the gut were also expressed in the filarial body wall, which is expected to be exposed to the immune system. Indeed, both *Bma*-iUGT and *Bma*-LAD-2 are

also found in the body wall but at dramatically lower levels. Still much of the filarial-specific IgE found in previously-exposed individuals is against the body wall of filaria. In addition, 4 excretory-secretory (ES) products were also found to be enriched in the gut; 3 of which were hypothetical proteins and the other was identified as a papain family protease (Bm1_18805). By their nature, ES products are exposed to the immune system of the host, and typically elicit a robust antibody response in the host

Further evidence that not all filarial intestinal proteins are hidden was demonstrated in a previous study by our lab. Basophils from mice that were 8-weeks infected with *L. sigmodontis* exhibited similar activation in the presence of *L. sigmodontis* soluble worm lysate versus soluble gut antigen [222]. In addition, *L. sigmodontis*-infected mice (8 weeks and 22 weeks post-infection) produced specific IgG and IgE to the gut antigen; though both antibody titers were significantly higher in response to whole worm lysate. Finally, this study showed that splenocytes treated with gut antigen underwent proliferation and produced cytokines. These findings indicate that there may be a pre-existing immune response in host organisms to soluble filaria intestinal antigen. We are most concerned about the pre-existing specific IgE to intestinal antigen. However, this is not an unexpected result and could be explained by proteolytic ES products released from the gut such as the previously mentioned protease papain. Indeed, there are a number of common allergens that exhibit proteolytic activity such as Der p 1 and Der p 2 (dust mite proteases), Pen c 13 (serine protease in mold), and Amb a 11 (cysteine protease in ragweed) to name a few [223].

With our study, we purposely selected proteins enriched in the intestine that were not ES products. This could explain the lack of specific IgE detected by LIPS against our 2 proteins. Had we used the full soluble portion of the *Brugia* gut, we would have likely detected an IgE signal as made evident by presence of specific IgE in *L. sigomodontis*-infected mice. We are prepared to perform this experiment in the future. Considering the pre-existing IgE response for total soluble gut antigen, a safe vaccine would most likely only consist of a single intestinal antigen or a combination of multiple intestinal antigens. Also, if one were dissecting the intestinal tract, harvesting enough soluble antigen from whole filarial intestine to produce a protective immune response would be extremely cost and time prohibitive. Furthermore, we demonstrated that this approach was not protective against filarial infection [222]. Though, this could be attributed to the possibility that any potential protective portion of the vaccine was not at a high enough concentration. Indeed, Vedi et al. showed that vaccination with *Brugia* body wall homogenate reduced adult worm burden by 25% whereas vaccination with a single antigen, a recombinant *Brugia* myosin heavy chain, reduced adult worm burden by 64% [99]. Therefore, we believe that deliberate selection of a few essential filarial proteins that are preferentially expressed in the intestine and not ES products has the highest chance of success in terms of vaccine efficacy and safety.

VACCINE CONSIDERATIONS

Our study identified *Bma*-iUGT and *Bma*-LAD-2 as possible vaccine antigens. However, consideration must be given to which expression system should be used. So far, post-translational modification prediction software

YinOYang and NetNGlyc identified potential glycosylation sites on most of our candidate proteins. Glycosylation plays an important factor in antigen recognition by the immune system [224]. And while there have been several *Brugia* antigens expressed in bacteria [97-100, 103, 111], we believe that the absence of this modification from antigen could result in the immune system targeting an epitope that is normally glycosylated during natural infection. In turn, lack of appropriate glycosylation could reduce the efficacy of the vaccine. Therefore, to ensure antigen glycosylation and better mimic what is seen during natural infection, a eukaryotic expression system should be used. Indeed, one eukaryotic expression system, *Pichia pastoris*, has been used to recombinantly express several *Brugia* antigens [225, 226]. A past study showed that one such recombinant antigen *Bm*-RAL-2, reduced adult worm burden by 42% in infected jirds that were immunized [225].

Another consideration is whether a *Bma*-iUGT vaccine antigen should have enzymatic activity. UGTs catalyze the transfer of UDP-glucuronic acid onto molecules, so it would not be unexpected to observe glucuronidation by active *Bma*-iUGT in immunized individuals. This could change the activity or binding interactions of extracellular proteins, which may result in adverse effects in vaccinated individuals. Indeed, recombinant *Na*-APR-1 (hookworm vaccine antigen) has been shown to digest host proteins, which led to the use of inactive *Na*-APR-1 as an effective alternative [114]. Though it can be expected that extensive testing in animal models would reveal whether this is a concern.

Finally, using a combination of *Bma*-iUGT and *Bma*-LAD-2 in a vaccine may be more protective than just a single antigen. Studies have shown a higher level

of protection when immunizing with a multivalent vaccine or fusion protein [86, 225]. Targeting both proteins may kill the adult filaria quicker because 2 essential intestinal functions are inhibited.

FUTURE DIRECTIONS

With regards to this study, we propose that future work involve characterization of these proteins. For *Bma*-iUGT, we suggest recombinantly expressing the protein in a eukaryotic cell line. We can then employ a commercially available UGT activity assay to measure the enzymatic activity of the recombinant. This assay uses proluciferin as the substrate whereby glucuronidation of proluciferin prevents its conversion to luciferin and subsequent ability to produce light. We are also ready to perform immunohistochemistry (IHC) on the *Brugia* intestine to determine the location of *Bma*-iUGT within the intestine tract and whether the protein is truly expressed in the plasma membrane. IHC of the siRNA-treated filaria would provide additional validation of expression knockdown.

For *Bma*-LAD-2, we could perform an immunoprecipitation of the protein to identify binding partners for its cytoplasmic tail. Knocking this protein down may have resulted in a stoichiometric change in proteins present resulting in the disruption of the epithelium. Elucidation of this interaction could reveal the pathway by which the intestinal epithelium is maintained. In addition, IHC of the protein would provide useful information about its location within the intestine. To investigate the possibility that hemolymph is leaking into the intestinal lumen after *Bma*-LAD-2 knockdown, we are prepared to perform an osmolarity analysis of the luminal contents in adult filaria.

Furthermore, we propose evaluating the macrofilaricidal activity of sulfinpyrazone and probenecid in Mongolian gerbils (jirds), which are permissive to *B. malayi* infection. Past studies have shown that concentrations similar to those that exhibit macrofilaricidal activity in our study are achievable in rabbits, rats, and mice [158, 227-229]. We believe that similar blood concentrations could be attained in jirds. If these animal studies prove successful, translation to human use will be fairly rapid given that these drugs are already FDA-approved.

Using recombinantly active *Bma*-iUGT, we could also employ high-throughput drug screening using commercially available drug libraries. We would analyze the ability of these compounds to inhibit glucuronidation by *Bma*-iUGT using a UGT activity assay. This may reveal additional drugs that could exhibit macrofilaricidal activity and possibly expand the tools available to treat filarial infections. Similarly, a high-throughput drug screen of recombinant *Bma*-LAD-2 may identify compounds that inhibit the homophilic binding of the protein. With this experiment, we would express the outer membrane portion of the *Bma*-LAD-2. We expect this protein, which normally binds to other IgCAMs, to aggregate. Therefore, we could evaluate the ability of a drug to inhibit aggregation of recombinant *Bma*-LAD-2. Any hits would then be investigated for *in vitro* macrofilaricidal activity.

We also intend to evaluate whether recombinantly expressed *Bma*-iUGT and *Bma*-LAD-2 are protective in animal models against *B. malayi* infection as well as other human filarial infections. Furthermore, we propose investigating whether vaccine using these antigens would be protective in a dog model of *D. immitis*

(heartworm). Though, we would first perform siRNA experiments with adult *D. immitis* worm to determine if *Bma*-iUGT is an essential protein for survival. Heartworm infects over 200,000 cat and dogs in the United States. As with LF, there are limited therapeutic options to treat heartworm. Currently, household pets receive a monthly prophylactic, which costs around \$75-100 per year per pet [230, 231]. Considering the 80 million dogs in the United States, administering prophylactic treatment nationwide has an immense financial impact, which could be alleviated by a protective vaccine.

There is also applicability of this study's findings to other helminth infections. Given the rise in drug resistance with *H. contortus*, targeting intestinal UGTs may be a viable solution. As previously mentioned, this class of proteins (UGTs) have been implicated in the metabolism of anthelmintics and therefore, if targeted, could reduce drug resistance. In addition, it is possible that UGTs are essential proteins for *H. contortus* and targeting the helminths with UGT inhibitors could result in worm death. Thus, drug efficacy studies using probenecid and sulfinpyrazone would be an intriguing prospect in *H. contortus*.

Finally, we could evaluate other intestinal proteins in *B. malayi* adult worms as possible therapeutic targets. We would employ the same approach used in this study. siRNA inhibition would be used to reduce target protein expression in adult filaria. We then would look for a phenotypic difference in worm viability and fecundity.

LF is still a major health concern worldwide, and while we have fairly effective drugs to treat the disease, the goal of eliminating LF may require more

time and resources than previously believed. We believe that new therapeutics that target the adult filaria may accelerate these elimination efforts. In this study, we identified 2 intestinal proteins in adult *B. malayi* worms that may serve as a novel drug and vaccine targets. Furthermore, we have identified 2 FDA-approved drugs that, if found effective in animal models, could be rapidly translated to human use.

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