TRICHURIS MURIS WHEY ACIDIC PROTEIN INDUCES TYPE 2 PROTECTIVE IMMUNITY AGAINST WHIPWORM

Neima Briggs

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TRICHURIS MURIS WHEY ACIDIC PROTEIN INDUCES TYPE 2 PROTECTIVE IMMUNITY AGAINST WHIPWORM

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TRICHURIS MURIS WHEY ACIDIC PROTEIN INDUCES TYPE 2 PROTECTIVE IMMUNITY AGAINST WHIPWORM

A

DISSERTATION

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Neima Briggs, B.S., D.T.M.
Houston, Texas

August 2018
This dissertation is dedicated in loving gratitude to my parents –

My mother, Samileh Mozafari
and
My father, Benjamin Hicks Briggs
Acknowledgments

This dissertation could not have been completed without the tremendous support of many colleagues and loved ones over the years. I am forever appreciative of their encouragement and insight throughout my years of graduate school.

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Human whipworm (*Trichuris trichiura*) infects approximately 1 in 15 people worldwide, representing the leading infectious cause of colitis and subsequent, inflammatory bowel disease (IBD). Current control measures focused on mass deworming have had limited success due to low drug efficacies. Vaccination would be an ideal, cost-effective strategy to induce protective immunity, leading to control of infection and transmission. Here we report the identification of whey acidic protein, a whipworm secretory protein, as a strong immunogen for inducing protective efficacy in a surrogate mouse *T. muris* infection model. The near full-length recombinant WAP protein (*r*"Tm-WAP49), as well as a single, highly conserved repeat within WAP (fragment 8) expressed as an Na-GST-1 fusion protein (*r*"Tm-WAP-F8+Na-GST-1), generate a strong T helper type 2 (TH2) immune response when delivered as subcutaneous immunization formulated with Montanide ISA 720. Oral challenge with *T. muris* infective eggs following vaccination led to a significant reduction in worm burden of 48% by *r*"Tm-WAP49 and 33% by *r*"Tm-WAP-F8+Na-GST-1. The cellular immune correlates of protection included significant antigen-specific production of TH2 cytokines IL-4, IL-9, and IL-13 by cells isolated from the vaccine-draining inguinal lymph nodes, parasite-draining mesenteric lymph nodes, and spleen in mice vaccinated with either *r*"Tm-WAP49 or *r*"Tm-WAP-F8+Na-GST-1. The humoral immune correlates included a high antigen-specific ratio of IgG1 to IgG2a, without eliciting an IgE-mediated allergic response. Immunofluorescent staining of adult *T. muris* with WAP antisera identified the worm’s pathogenic stichosome organ as the site of secretion of native *Tm*-WAP protein into the colonic mucosa. While both *r*"Tm-WAP49 or *r*"Tm-WAP-F8+Na-GST-1 have high
purity and stability properties by analytical analysis, rTm-WAP49 has a more complex biophysical profile, with oligomerization to dimers and trimers through intermolecular disulfide bond formation. Recognition of rTm-WAP49 by endemic patient serum samples suggests that conserved epitopes may exist between *T. muris* and *T. trichiura* (or possibly non-*Trichuris* nematodes) derived WAPs. To our knowledge, this is the first study identifying a promising immunogen for further investigation of its vaccine potential against *T. muris* and eventually against *T. trichiura*. 
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| Abbreviation | Description |
|--------------|-------------|
| ADCC         | Antibody-dependent cell-mediated cytotoxicity |
| BCA          | Bicinchoninic acid |
| BLAST        | Basic Local Alignment Search Tool |
| CAP          | Cysteine-rich secretory proteins, antigen 5, and pathogenesis-related |
| CD           | Circular dichroism |
| CFA          | Complete Freund’s adjuvant |
| DALY         | Disability-adjusted life year |
| DLS          | Dynamic light scattering |
| ES           | Excretory and secretory |
| HPLC         | High performance liquid chromatography |
| IBD          | Inflammatory bowel disease |
| IFA          | Incomplete Freund’s adjuvant |
| IFN          | Interferon |
| IHC          | Immunohistochemistry |
| IL           | Interleukin |
| IMAC         | Immobilized metal affinity chromatography |
| MDA          | Mass drug administration |
| NTD          | Neglected tropical disease |
| SCFR         | Mast/stem cell growth factor receptor |
| STH          | Soil-transmitted helminth |
| T reg        | Regulatory T cell |
| TGF          | Transforming growth factor |
| TH1          | T helper type 1 |
| TH2          | T helper type 2 |
| TNF          | Tumor necrosis factor |
| UPLC         | Ultra performance liquid chromatography |
| WAP          | Whey acidic protein |
| WHO          | World Health Organization |
Chapter I

Introduction
Portions of this chapter are based upon:

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   • Referenced through the dissertation as (1).

B. Briggs, N.*, J. Weatherhead*, K. J. Sastry, and P. J. Hotez. 2016. The Hygiene Hypothesis and Its Inconvenient Truths about Helminth Infections. PLoS Negl. Trop. Dis. 10: 1–12.
   • Referenced through this dissertation as (2).

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This is an Accepted Manuscript of an article published by Taylor & Francis in [Expert Review Vaccines] in [March 2014], available online:

[https://www.tandfonline.com/doi/full/10.1586/14760584.2014.872035]”

B. “PLOS publishes all work under a Creative Commons CC BY license, which grants copyright to the authors and allows anyone, anywhere to share/adapt the work for any reason, so long as proper attribution is given.”
1.1 Soil Transmitted Helminths: the “Unholy Trinity”

The three major soil-transmitted helminths (STHs) include, whipworms (*Trichuris trichiura*), hookworms (*Necator americanus* and *Ancylostoma duodenale*), and roundworms (*Ascaris lumbricoides*). These parasitic worm infections plague the world’s most impoverished billion people (3). The STHs fall into the broader grouping of neglected tropical diseases (NTDs), comprised of 20 diseases classified together by the World Health Organization (WHO) due to their longstanding public health disregard, despite affecting one-sixth of the global population (4). The STHs are the most prevalent infections among the NTDs and malaria (Figure 1) (5). According to the Global Burden of Disease Study of 2016, approximately 435 million people are infected with the whipworm, 800 million people are infected with the roundworm, and 450 million are infected with hookworms, the later mostly include *Necator americanus* infections (6).

![Figure 1. Global prevalence of neglected tropical diseases and malaria with age in 2016. This figure was generated with data from the Institute for Health Metrics and Evaluation at the University of Washington (5).](image-url)
Recent estimates indicate that STH infections are responsible for 5.19 million disability adjusted life years (DALYs), which leads all NTDs (7). These intestinal parasites cause chronic, debilitating disease including iron deficiency anemia, asthma, vitamin deficiency, abdominal pain, colitis, and dysentery, leading to growth failure and impaired cognitive development (8). The STH infections perpetuate poverty within endemic communities due to economic suppression from decreased education accrualment, loss of workforce, and cost of treatment (4, 9, 10). The NTDs, including STHs, are considered ancient diseases, with references in numerous ancient texts, including the Talmud, Bible, and the writings of Hippocrates (11). The STH eggs have even been identified in the mummified remains of human stool (3). It is theorized that over these millennia of host-parasite interactions, helminths evolved complex immune evasion capabilities and subtle clinical presentations to subvert detection and expulsion in humans (2).

Our research group within the Texas Children’s Hospital Center for Vaccine Development has a long-standing interest in the NTDs, with a focus on vaccine development. A vaccine can serve as a low-cost and long-term solution to curb these poverty-promoting diseases. This dissertation will focus on one STH, *Trichuris trichiura*, with an emphasis on the identification and characterization of antigens along with investigating their immune-mediated mechanisms of protection in a murine model.

### 1.2 Life Cycle and Pathogenesis

*Trichuris trichiura*, known as the common whipworm of humans and nonhuman primates, is a parasite of the superfamily Trichinelloidea (1). *Trichuris* species are common parasites of the mammalian kingdom, but are relatively host-specific (12). For purposes of vaccine development, there is no established laboratory animal model for *T. trichiura* because of its specificity restricted to humans and some non-human primates (12, 13). Fortunately, the closely related *Trichuris*
*muris* parasite, specific to mice, has emerged as a useful surrogate model to screen for antigen immunogenicity and efficacy (14).

In both cases of *T. trichiura* and *T. muris*, infection occurs through direct ingestion of embryonated eggs from contaminated food, soil, water, or via soiled hands (15). Ingested whipworm eggs hatch into larvae in the terminal ileum and caecum due to interactions with the host microbiome (16). At the cecum, the helminth embeds its anterior portion within the colonic epithelium and matures into adult worms in 1 to 3 months, in case of *T. trichiura* (17) or in 35 to 42 days, in case of *T. muris* (14). In humans, *T. trichiura* can grow to approximately 3-5 cm in length, with the females slightly larger than the males and both living for 1-3 years, although there are reports of cases where they have survived substantially longer (17, 18).

*Trichuris* spp. cannot complete its lifecycle within the host and must pass its unembryonated eggs from the posterior, intraluminal end of the parasite out of the animal with the passing bowel movement (18). An infected human can harbor dozens to thousands of adult whipworm (19). A single *T. trichiura* female can release 3,000 to 20,000 barrel-shaped ova a day, leading heavily-infected people to pass millions of eggs through their stool a day (18). The WHO defines the intensity (or burden) of infection based on the number of eggs per gram of feces, with light, moderate, and heavy infections quantified as 1-999, 1,000-9,999, and greater than 10,000 eggs, respectively (18). The intensity of infection has been shown to correlate with disease severity (19). In the soil, the zygote egg development goes through an advanced cleavage stage, ultimately becoming embryonated, and infectious, after 10-14 days (19). The egg can remain viable for several years in soil and withstand high variations in temperature, allowing the parasite to subsist beyond tropics and into temperate climates (18, 19).

Within the phylum Nematoda, both *Trichuris* whipworms and *Trichinella*, parasites causing trichinosis, belong to the order Trichocephalida (*Figure 2*) (20), which has as a hallmark, a unique parasite organ known as the stichosome (*Figure 3*) (14, 21). The stichosome is thought to enable burrowing of the anterior portion of the worm into the mucosal epithelium of the host to
provide the parasite an anchor preventing physical expulsion (21). Thus, the whipworm has both an intercellular existence at its anterior end, as well as an extracellular, intraluminal posterior end. The stichosome holds reservoirs of secretary granules containing macromolecules speculated to be necessary for host invasion into the colonic mucosa through pore-formation, enabling feeding, and modulation of host immune response (21–23). Whipworms cultured in vitro release excretory and secretory products (ES) that are a rich source of antigens that this dissertation has used as a source for testing immunogenicity and efficacy towards future evaluation as potential vaccine candidates.

Figure 2. Phylogenetic analysis of *Trichuris* spp. and the major human and zoonotic STHs. Evolutionary tree of *T. trichiura* and *T. muris* (green clade) within the phylum Nematoda.
1.3 Impact of Trichuriasis on Host Inflammation in the Intestines

Associated with the public health impact of human soil-transmitted helminth infections, there is a profound level of global disability that arises from the pathologic sequelae of helminth infections translating into widespread mucosal immune and microbiome dysregulation and contributing to inflammatory diseases, such as asthma and inflammatory bowel disease (IBD) (2, 24). For example, *Ascaris* larval invasion of the lung may induce asthma and other allergic pulmonary consequences, while the *Trichuris* stichosome invasion of the colonic mucosa ultimately results in chronic colitis, a suspected driver of IBD. Although there are limited clinical...
studies reporting IBD from rural helminth-endemic areas, underreporting of disease may be due to limited diagnostic resources (2). In an Ecuadorian cohort of over 2,400 children in Esmeraldas province, where 28.6% of children had at least one documented soil transmitted helminth infection, 25.9% of children had wheeze, 15.2% had skin test reactivity to an aeroallergen, and 17.7% had an episode of eczema, suggesting a significant presence of allergic disease (2, 25). *T. trichiura* specifically attaches to the colon, where it can induce colitis, IBD, *Trichuris* dysentery syndrome, and rectal prolapse, especially in heavy infections (26, 27). The mechanisms responsible for these sequelae are likely a combination of direct tissue invasion by the adult parasite, together with a strong host inflammatory response. Through such processes, long-standing trichuriasis, similar to IBD, leads to growth suppression and cognitive impairments (28, 29). These sequelae can sometimes resolve after treatment, resulting in catch-up growth (30).

Use of *T. muris* as a model for of human IBD, has led to the identification of several key genes and immune correlates associated with susceptibility and severity (31, 32). Recent studies have shown that *T. muris*-infected colonic tissue histologically resembles established mouse models of IBD, with a defective epithelial barrier and a dominant TH1 immune infiltrate, leading to impaired intestinal mucosa homeostasis (32). Resistin-like molecule (RELMβ)—a protein secreted from goblet cells after local tissue damage triggers a proinflammatory cytokine milieu, with IFN-γ and tumor necrosis factor-alpha (TNF-α) cytokines driving the TH1 immune infiltration, leading to the chronic colonic pathology in both trichuriasis and IBD (33, 34). Mouse models of chronic trichuriasis result in massive crypt hyperplasia because of intra-epithelial lymphocytosis in the large intestines, hypothesized to be driven by a 43 kDa *T. muris* interferon-gamma (IFN-γ) homologue protein (35, 36).

Interleukin 18 (IL-18), a key TH1 immune regulator of intestinal homeostasis, is overexpressed in the human large intestine during both Crohn’s disease and in *T. muris*-infected mice (37–39). Importantly, IL-18 production in *T. muris*-infected mice showed direct suppression of critical TH2 cytokines, IL-4 and IL-13, necessary for worm expulsion (37). Nowarski and
colleagues showed that overexpression of IL-18 led to significant mucosal barrier dysfunction, including epithelial goblet cell hyposcretion of protective mucins and other essential proteins for barrier integrity (40).

The clinical, immunological, and histological homology, as well as genetic susceptibility, between *Trichuris*-induced colitis and both Crohn’s disease and ulcerative colitis warrants further investigation of *Trichuris* colitis as an environmental driver of IBD worldwide. For the purposes of this dissertation, we used several of the highlighted immune drivers of *Trichuris* colitis to evaluate the immune correlates of immunization-induced protection.

1.4 Helminths and the “Hygiene Hypothesis”

By some accounts, beginning in the late 20th century there was an increase in the reported cases of inflammatory and autoimmune diseases worldwide, such as asthma, inflammatory bowel disease (IBD), food allergies, and multiple sclerosis (MS) (41). This phenomenon has been linked to the evolution of a “westernized” lifestyle, declining family size, improved household amenities, higher personal cleanliness, and reduced cross infection within affluent communities (42). From this notion the “hygiene hypothesis” evolved, suggesting a reduction in microbial exposures, secondary to hygienic conditions, impeded the proper maturation of the immune system. The hygiene hypothesis has become accepted by many in the global scientific community and has evolved to associate multiple variables as either protective or contributory in the development of inflammatory diseases. Identified environmental factors that have been incorporated into the hygiene hypothesis include variations in microbial exposure, parasites, diet, medications, lifestyle behaviors, sanitation, occupations, and pollutant exposures (41, 43). A branch of this hypothesis points at parasitic helminths, which according to some have been nearly eradicated from the westernized regions, as the key immune modulator necessary for proper immune development.
Despite the severe negative consequences of trichuriasis and helminth infections highlighted above, some studies present a counterview of generalized human helminth infections as potentially protective against the development of autoimmune and inflammatory diseases due to their significant immune modulatory properties within a host. Helminths can maintain host evasion for years through an array of mechanisms that down-regulate host innate and adaptive responses. These include immunomodulatory proteins containing host-related glycans and lipids that direct cytokine mimicry and interference, non-protein signature molecules that result in immunosuppressive host cytokine release, and direct interference of antigen presentation. The presence of T regulatory cells (T reg) along with immune modulatory cytokines Interleukin-10 (IL-10), transforming growth factor (TGF)-β, (44) and, at basal levels, Interleukin-18 (IL-18) (37) promote helminth survival while quelling the host inflammatory response.

Ironically, despite the direct association of trichuriasis to IBD, there have been clinical trials with live helminths that aim to harness their anti-inflammatory properties as a treatment modality for patients with IBD. Initially, two open-label studies by Summers et al. showed that pig whipworm *T. suis* therapy for the treatment of IBD correlated to a remission rate of around 70%, helping ignite excitement for helminth therapy in human beings (45, 46). However, two blind, placebo-controlled Phase 2 clinical trials in the US with 250 patients, (TRUST-1, trial identifier NCT01576471) and in Europe with 240 patients, (FALK, trial identifier NCT01279577) using *T. suis* eggs for moderate-to-severe Crohn’s disease were terminated in 2013 due to a lack of efficacy, determined by both the disease activity index and remission rates (47, 48). Additionally, a Cochrane review, which is a meta-analysis of all relevant clinical trials and data on a health care intervention, found insufficient evidence of safety or efficacy of *T. suis* therapy to induce inflammatory bowel disease remission (49).

To date the overwhelming scientific data point both to the harmful health effects of helminths, and the ability of helminth infections to impair economic development. Given the known risks of live helminth inoculations, research highlighting helminth immunomodulatory
properties should direct future research towards identifying specific helminth-derived molecules that could be developed into therapies. Further, it’s important not to diminish the role of helminths in inciting and exacerbating inflammatory diseases globally, including in wealthy countries such as the US. Therefore, prioritizing helminth elimination efforts globally through control tools such as anthelminthic drugs and vaccines remains paramount (2).

1.5 Current Therapeutics: Limitations and Need for Innovation

Global control of soil-transmitted nematode infections is based on annual (or sometimes twice-annual) mass drug administration with a single-dose of albendazole (400 mg), mebendazole (500 mg), tribendimidine (400 mg), pyrantel pamoate (10 mg/kg), or levamisole (2.5 mg/kg) for children between the ages of 1 and 14 who live in areas where the prevalence of these infections exceed 20% (50). The World Health Organization (WHO) has identified millions of children in every region of the world in need of treatment, although the call for treatment is substantially higher in the regions of Africa and South-East Asia due to increased prevalence (Table 1). The WHO estimates that in 2016, 69.5% of the world’s children who require mass treatment for STH, actually received their medication (51). The major goal of global deworming is morbidity reduction, especially to promote childhood growth and cognition, although increasingly there is interest in determining whether deworming itself might eventually lead to elimination of soil-transmitted helminthiases (52). Currently, pregnant women do not consistently receive anthelminthic treatments, due to unknown teratogenicity, leading to malnutrition and risks of fetal complications (53).
Aside from the low treatment coverage for children, a situation being remedied through expanded donations of albendazole and mebendazole and new global policies including a recent World Health Assembly resolution (54), there are concerns that pediatric mass drug administration alone may not be sufficient for global control of soil-transmitted nematode infections, and certainly not global elimination (55).

The major reasons for why annual deworming may not be successful as an isolated intervention are:

- High rates of post-treatment reinfection, especially in areas of intense transmission, which might require a higher frequency of deworming, in some cases every four months or more frequently (56).

| WHO Region                  | Number of children requiring albendazole or mebendazole | Percentage of worldwide population requiring treatment |
|-----------------------------|--------------------------------------------------------|--------------------------------------------------------|
| African (AFRO)              | 296.5 million                                          | 33.8%                                                  |
| Americas (PAHO)             | 48.0 million                                           | 5.4%                                                   |
| South-East Asia (SEARO)     | 371.0 million                                          | 42.4%                                                  |
| Europe                      | 4.3 million                                            | 0.5%                                                   |
| Eastern Mediterranean (EMRO)| 80.5 million                                           | 9.2%                                                   |
| Western Pacific (WPRO)      | 73.3 million                                           | 8.4%                                                   |
| Globally                    | 874.5 million                                          | 100%                                                   |

Table 1. Geographic distribution of children who require regular deworming. This figure was adapted, with permission, from reference 1.
A systematic review of the 5 available drugs (albendazole [400 mg], mebendazole [500 mg], tribendimidine [400 mg], pyrantel pamoate [10 mg/kg], or levamisole [2.5 mg/kg]) against STHs revealed that none of these anthelminthics at the current single-dose regimen provided a satisfactory cure rate against trichuriasis (55, 57, 58). On the other hand, a single dose of albendazole, mebendazole, or pyrantel pamoate provided greater than 88% cure rates against *A. lumbricoides* (57). Similarly, a single dose of albendazole or tribendimidine provided 72% and 77.8% cure rate against hookworm, respectively (57, 59). Thus, these anthelminthics are poorly effective against trichuriasis with cure rates for the recommended single dose regimens at, 28% for albendazole, 36% for mebendazole, 23.1% for tribendimidine, 28.1% for pyrantel pamoate, and 8.6% for levamisole (57). Therefore, preventive chemotherapy has the least impact on human whipworm infection relative to other STHs. Yet another problem for trichuriasis are high rates of post treatment reinfection, especially without clean water and sanitation (60). As a consequence, the global prevalence of trichuriasis has decreased by just 16.7% between 2005-2015 (61). In that same time period, hookworm disease had a similar decrease by just 14.3%, whereas ascariasis decreased by 36.5% (61).

The potential for drug resistance, which has already emerged in veterinary medicine for horses and cattle mass treated with benzimidazoles and macrocyclic lactones (57).

Failure to fully consider the transmission dynamics of soil-transmitted nematode infections, which affects both adult and pediatric populations and would require targeting of both populations for a truly effective elimination strategy (55).

Failure to co-implement aggressive programs of sanitation and access to clean water in order to complement deworming (62).

A recent Cochrane analysis has questioned the benefits of mass treatments and deworming based on lack of consistent evidence for its beneficial impact on nutrition, anemia, and school
attendance or performance (63). It is likely that some of the factors outlined above, i.e., rapid re-infection and lack of drug efficacies, have a role in the Cochrane findings. However, the Cochrane analysis also did not differentiate between the effects of individual nematode species or their differential drug susceptibilities (54). The Cochrane analysis partly blurs these findings by treating all soil-transmitted nematode infections and their treatments as equivalent (54). Nevertheless, there is an urgent need to improve effectiveness and efficiencies of global deworming to achieve key milestones and Millennium Development Goals. Approaches to improve global deworming were recently suggested (55). They include adding ivermectin, mebendazole, or oxantel pamoate to albendazole in areas where high levels of trichuriasis transmission occurs (55, 64, 65).

To summarize, human trichuriasis represents one of the world’s leading causes of IBD, especially among children living in poverty. The current approach towards global helminth control, namely regular deworming, has the least effect for human trichuriasis compared to other soil-transmitted helminth infections.

A cost-effective practical approach to the global control and elimination of soil-transmitted nematode infections is the development (and distribution) of anthelminthic vaccines that can be used in conjunction with chemotherapy.

1.6 Status of *Trichuris* Antigen Discovery

Whipworm has not been studied as extensively as hookworm or roundworm with respect to the cloning, expression, and evaluation of recombinant antigens for immunogenicity. Hence the field is at an infancy stage for a vaccine development pipeline. The concept of a human vaccine targeting trichuriasis was first proposed by Prof. Derek Wakelin and his colleagues in the 1990s (66). Since then, most of the successes with *T. muris* infection in mice as the model have been associated with either the parenteral injection of adult-stage worm antigens, especially those
containing stichosome-derived proteins for determining efficacy, or their administration via mucosal routes (summarized in Table 2).

- Worm extracts. Homogenized adult *T. muris* antigens, either emulsified in complete Freund’s adjuvant (CFA) for subcutaneous immunization, or combined with cholera toxin for oral administration have been administered to several different mouse strains (66). The mucosal IgA response was enhanced significantly by oral vaccination to induce protection in BALB/c and C57BL/10 mice, but not in B10.Br mice for unknown reasons (66). In contrast, subcutaneous administration provided enhanced protective immunity in all three mouse strains (66).

- ES Antigens. Stichosome-related adult-stage *Trichuris spp.* excretory and secretory (ES) products have been identified as potential sources of protective antigens (67), although some stichosome proteins remain stored in the organ and are not actually secreted (68). Near-sterile immunity has been observed following immunization with adult ES products in multiple susceptible and resistant strains, in particular when co-formulated with complete Freund’s adjuvant (CFA) or incomplete Freund’s adjuvant (IFA) (66, 69–72). The protection elicited in susceptible AKR mice included reductions in parasite egg shedding and was associated with a TH2 peripheral lymph node responses to in vitro ES stimulation, namely IL-4 and IL-13 secretion into the cellular supernatant (69). Three pore-forming proteins derived from the ES were identified and these include: TM43 from *T. muris*, (73), and TT52 (23) and TT95 (21) from *T. trichiura*. However, evaluation of immunogenicity or efficacy of these proteins has not been reported to our knowledge.

- *Trichinella* antigens. Murine studies have identified cross-reactivity and cross-immunity between *Trichuris* and its phylogenetically related nematode genus, *Trichinella*, proteins (74). Using ELISA, immunoprecipitation and immunoblotting, it was demonstrated that *T. trichiura*-infected mice had cross-reactive antibodies to *T. spiralis* (74). Shared stichocyte antigens have also been noted (75). Among the defined candidate antigens identified
from *T. spiralis* that provide partial protection are an aminopeptidase (76), serine proteases (77–79), parasite-derived cytokines (80), paramyosin (81), heat shock protein (79) and secreted antigens of 87 kDa, 53 kDa, and 43 kDa size with unknown function (81–83).
| Antigen       | MW    | Functions                      | Paraseite stages | Protective efficacy                      | Reference |
|--------------|-------|--------------------------------|------------------|------------------------------------------|-----------|
|              |       |                                |                  | Adjuvant | Worm reduction (HR, LR) |
| **Trichuris muris** |
| T. muris adult worm extracts | Various | Unknown | Adult | CFA | HR(BALB/c) = 99.2% LR(CS7BL/10) = 97.8% LR(B10.BR) = 41.4% | (66) |
| T. muris adult ES       | Various | ES products    | Adult | CFA | LR(AKR) = 100% | (69) |
| **Trichinella spirals** |
| TsAP         | 54.7 kDa | Aminopeptidase   | Larva | CFA/IFA | 8.1% (adult worm) 59% (muscle larva) | (76) |
| TspSP-1.2    | 35.5 kDa | Serine protease    | Adult, ES | None | 34.92% (adult worm) 52.24% (muscle larva) | (79) |
| rTs-Adsp     | 47 kDa  | Serine protease    | Adult | Alum | 46.5% | (78) |
| pVAX1-Tsmif-Tsmcd-1 | 59 kDa  | Macrophage inhibitory factor & cysteine protease inhibitor | All | None | 23.17% | (80) |
| rTs-Pmy      | 102 kDa | Binding to C8, C9 (complement) | Adult, Larvae | ISA50 | 21.8% (muscle larva) 33.4% (induced by epitope 88-107aa) | (81) |
| Ts87         | 87 kDa  | Surface antigen     | Adult | DNA carried by *S. typhimurium* | 29.8% (adult worm) 34.2% (muscle larva) | (82) |
| Ts53         | 53 kDa  | Secreted glycoprotein | Adult, larva, ES | ND | ND | (83) |
| Ts adult ES  | various | ES products | Adult | ND | 98.4% (adult worm); 82.9% (muscle larva) | (84) |
| Ts-Hsp70     | 70 kDa  | Heat shock protein   | Adult | CFA | 37% | (79) |

ES: Excretory and secretory; CFA: Complete Freund’s adjuvant; HR: High responder; IFA: Incomplete Freund’s adjuvant; ISA50: Incomplete Seppic adjuvants 50; LR: Low responder; MW: Molecular weight; ND: Not done.

**Table 2. Summary of major *Trichuris muris* and *Trichinella spirals* antigens discovered.**

This figure was adapted, with permission, from reference 1.
As outlined above, there are no defined *T. trichiura* antigens identified for potential application towards vaccine development. Thus, there is still a need to identify *T. trichiura* antigens for evaluating immunogenicity and potential efficacy in suitable animal models. One approach to identify a vaccine antigen would be to select the major *Trichinella* antigens with the highest *Trichuris* spp. homology and test each in a *T. muris* animal model for protective immunity. Yet another approach would be to screen the protective *Tm*-ES or *T. muris* homogenate for immunodominant antigens. However, given the complexity of macromolecule composition is simpler in stichosome-derived ES compared to whole worm extracts, the former may represent a more attractive source for purposes of antigen discovery. Selected antigens can be cloned and expressed in bacteria (*E. coli*), yeast, or baculovirus vector platforms.

Given the high degree of homology at the gene and protein levels for several antigenic sequences between *T. trichiura* and *T. muris*, we selected *T. muris* adult ES products for identifying proteins that could be cloned and expressed as recombinant protein immunogens. Since the adult *T. muris* ES induces near-sterile immunity against infective egg challenge (69), we generated and used the protective *Tm*-ES antisera to immunoscreen the *T. muris* adult stichosome cDNA library to identify targets for vaccine development.

1.7 Immune Correlates of Protection Against Whipworm

Immunological protection against whipworm will likely depend on combined humoral and cellular type 2 immune response directed against one or more molecules found in *Trichuris* ES, as highlighted in the human and animal model studies reviewed above. Although sera from immunized mice can confer protection via passive transfer, if the recipient mouse is immunosuppressed, sera alone is not protective (67). A cellular component is believed to be
necessary to induce a resistant (type 2 immunity) vs susceptible (type 1 immunity) response against *T. muris* infection (14, 85).

Of critical significance to mounting type 2 immunity are T helper type 2 (TH2) cytokines IL-4, IL-9, and IL-13 (14, 86). In this regard, it is well-established that the TH2 cytokine IL-4 is a critical mediator for enhancing antibody responses (87), whereas IL-13 is recognized for its role in promoting worm expulsion through goblet cell expansion, mucin production, and smooth muscle hypercontractility. Similarly, IL-9 mediates smooth muscle hypercontractility, as well as tissue mast cell maturation (88, 89).

Both IL-4 and IL-13 bind to the IL-4Rα subunit to induce STAT6 signaling pathway and subsequent TH2 responses. Stat6-deficient mice splenocytes do not exhibit IL-4 secretion in response to *in-vitro* stimulation with UV-inactivated HSV-1 (90), have reduced systemic IL-4 and IL-5 levels to OVA antigen challenge (91), impaired ability of T lymphocytes to differentiate into TH2 cells (92), and no detectable IgE levels (91). Consequently, STAT6−/− mice develop both acute and chronic *T. muris* infections. The redundancy in IL-4 and IL-13 binding to IL-4Rα, in part explains why IL-4 deficient mice have reduced, but still partial resistance (89). Interestingly, whereas complete IL-4Rα ablation renders high susceptibility to helminth infection, the selective deficiency of IL-4Rα on T cells has no impact on worm burden (89). Rather, T cells may be critical for the production, rather than recognition, of IL-4, because T helper follicular cells induce IgG1 class switching of B cells via IL-4 (87). Although IL-5 is key for the expulsion of other geohelminth infections, predominately through downstream activation and recruitment of gut eosinophils (93), ablation of IL-5, and lack of subsequent eosinophilia, had no effect on the development of protective immunity against *T. muris* (94). Similarly, ablation of mast cells through blocking of mast/stem cell growth factor receptor (SCFR), c-kit, led to no change in protective immunity (94). This suggests that neither eosinophils nor mast cells play a key role in *T. muris* resistance.

In general, TH2-driven protective immunity to *T. muris* infection is most closely associated with elevated parasite-specific serum IgG1 (14). Selby and Wakelin demonstrated that passive
transfer of serum or adoptive transfer of lymphoid cells conferred increased resistance to *T. muris* infection (67). This transfer of protective serum, however, only provided protection when administered at the time of infection, and even then, the degree of protection was variable (67). Wakelin later demonstrated that immunosuppressed mice receiving serum transfer showed no protection against challenge, suggesting that a cellular component in recipient mice is necessary to mediate expulsion (95). To determine whether associated humoral protection was through antibody-dependent cell-mediated cytotoxicity (ADCC) against *T. muris*, Betts and Else evaluated FcyR-deficient with a resistant background (BALB/K) and found no change in immunity or parasite expulsion. This suggests that ADCC may not play a major role in *T. muris* protection.

1.8 Trichuriasis animal model

Protective immunity to *T. muris* in mice is strongly influenced by host genetics (96). Resistant “high responder” inbred mouse strains, such as BALB/c, develop a TH2 cytokine response (IL-4, IL-9, and IL-13) to *T. muris* infection with a corresponding rise in parasite-specific IgG1 antibodies, leading to expulsion of worms and complete resistance to reinfection for at least 60 days (1, 14, 97). Therefore, BALB/c mice are the primary model of choice for studying natural host resistance to *T. muris*. On the other hand, B10.BR, C57BL/6, and AKR mice serve as effective “low responder” efficacy models to evaluate candidate antigens, as they develop type 1 immune response to *T. muris* challenge, leading to high-burden disease, yet develop a protective type 2 immune response, when vaccinated with protective antigens such as *T. muris* excretory and secretory products (*Tm*-ES) prior to challenge (66, 69, 75). Stat6-deficient mice, which have an almost exclusive TH1 immune response to infection, are commonly used as a *T. muris* maintenance model, as adult *T. muris* only survive for 2-3 days ex vivo.

Previous studies in our lab tested multiple mouse strains and found the AKR mice from Jackson Labs are the most consistent model for acute, high dose infection (e.g. 300 embryonated
eggs). On the other hand, B10.BR and C57BL/6 are better models of chronic infection by a single low-dose or repeated low-doses, known as a trickle-infection, which may better simulate a natural exposure to *Trichuris spp.* eggs (14). Since female mice exhibit a stronger TH2 response and have increased resistance to *T. muris* (98), male AKR mice were used for these initial studies. Eventually, any identified candidate antigens would need to be evaluated for immunogenicity and efficacy in female mice.

1.9 Vaccine Route and Adjuvant

Both immune response and epitope recognition of immunogens can vary substantially with different routes of administration and co-formulated adjuvants. Mucosal vaccination (*i.e.* oral, sublingual, or intranasal) is an obvious option for gut pathogens, such as STHs, as this route induces the most substantial gut immune response (99). Additionally, it has a major advantage over parenteral immunizations because of administration ease in low resource populations that STHs predominately afflict, as seen with the oral polio vaccine. Intranasal immunization with recombinant *As*14 protein coupled to cholera toxin B subunit induced a 64% reduction in worm burden against *Ascaris suum* in BALB/c mice (100). However, with *T. spiralis* equal efficacy (~55%) was observed when muscle larval homogenate formulated with cholera toxin was delivered by oral administration or the same homogenate formulated with CFA for subcutaneous delivery in the *T. spiralis* animal model (66). In the *T. muris* models, however, Robinson and colleagues showed that secretory IgA induced by oral immunization using *T. muris* adult homogenate with cholera toxin adjuvant only gives partial protection in certain mice strains (BALB/c & C57BL/10), whereas subcutaneous immunization using the same antigen with CFA, induces enhanced protection in all mouse strains (BALB/c, C57BL/10, B10.BR) tested and is closely associated with high systemic IgG1 titers (1, 66). In humans, oral vaccination against gut pathogens *S. enterica* serovar Typhi and *Vibrio cholera* led to variable induced immunity in
endemic populations due to underlying variability in the intestinal microbiota, which does not affect parenteral administration (101).

As described earlier, protection against whipworm is closely associated with host TH2 response. Thus, an adjuvant that direct hosts immunity in the direction of a TH2 response would be a logical choice for co-formulation. Although CFA is the primary adjuvant of selection, due to its high toxicity it would not translate for human use and is less used in animal testing (102). Montanide ISA 720 is a squalene-based water-in-oil emulsion used in over 50 clinical trials (103, 104). Montanide ISA 720 has been well-demonstrated to induces a strong and long-term combined TH2/TH1 response (102). Despite the dual TH2/TH1 response, Montanide ISA 720 has been used in multiple efficacious helminth preclinical animal models, including in schistosomiasis and the closely related *Trichinella spiralis* (102–104). Additionally, aluminum-based adjuvants, such as aluminum phosphate and aluminum hydroxide, are TH2-biased adjuvants widely used in humans and animals, including anthelminthic vaccines (105).

1.10 Conceptual Framework and Hypothesis

The long-term goal of our lab is to develop a vaccine against *T. trichiura*. We exploited the high degree of genetic sequence homology between *T. trichiura* and *T. muris* pathogens to identify immunogenic *T. muris* stichosome-derived proteins and investigated their ability to elicit protective immunity against intracellular parasite establishment.

The central hypothesis of this dissertation is that *Trichuris* spp. stichosome secretory proteins will induce protective type 2 immunity in a murine model.

To test this hypothesis, the following specific aims were carried out:

1. Identification of *T. muris* stichosome-derived antigens and expression as recombinant proteins.
1. Identify highly abundant antigens in the *T. muris* stichosome cDNA expression library using sera from mice immunized with the *Tm*-ES.

b) Express and purify identified antigens from the *T. muris* stichosome cDNA expression library as recombinant proteins.

2. **Evaluate the immunogenicity and efficacy of *T. muris* recombinant proteins in a murine model.**

a) Determine the protective efficacy of recombinant proteins corresponding to the two highly abundant *T. muris* stichosome antigens in the AKR mouse model.

b) Determine the humoral immune responses induced by the *T. muris* stichosome antigens by ELISA analysis.

c) Determine the T helper cell responses in spleen, draining lymph nodes, and mesenteric lymph nodes induced by the *T. muris* stichosome antigens using Luminex® analysis.

3. **Biophysical characterization and localization of a protective *T. muris* protein.**

a) Determine the location of expression of the native protein in adult *T. muris*.

b) Assess the homogeneity and purity.

c) Determine the secondary structure, thermostability, and post-translational modifications.

4. **In vitro evaluation of human immune recognition of protective *T. muris* protein.**

a) Determine the prevalence of *T. trichiura* in an endemic region of Honduras by qPCR.

b) Evaluate serum IgG and IgE recognition of the protective *T. muris* protein in endemic participants by ELISA.

c) Evaluate human T cell responses to the protective *T. muris* protein using Flow Cytometry and Luminex® analysis.
Chapter II

Identification of *T. muris* stichosome-derived antigens and expression as recombinant proteins.
2.1 Introduction

The current method to control trichuriasis by mass drug administration (MDA) employing albendazole or mebendazole does not reduce the overall prevalence or associated morbidity (106). Though vaccination is a long-term and cost-effective strategy to induce protective immunity, possibly leading to the control of infection and transmission, there are no commercially available anthelminthic vaccines (1). However, clinical trials are testing recombinant protein-based vaccines against schistosomiasis (Sm-TSP-2 and Sm-14) (107) and hookworm (Na-GST-1) (108). While DNA and mRNA vaccines hold promise as alternative strategies, they have yet to be successful in human trials (109, 110). Furthermore, recombinant protein vaccines against infectious diseases have a good safety record and low production costs (111).

Currently, there are no approved vaccines for human whipworm (1). Potential sources of whipworms antigens include: the whole worm extract (63, 66), the worm’s excretory and secretory (ES) products (21, 23, 67–69, 73, 112), and defined candidate antigens corresponding to the closely related Trichinella spiralis (76–83) (Summarized in Chapter 1, Table 2). The helminths’ anterior-end organ, known as a stichosome, excretes products that facilitate invasion and maintenance of the parasite into the host’s gut epithelia (14, 22). The T. muris ES, thought to predominately originate from the stichosome, can be collected from the media of in-vitro cultured helminths. Near-sterile immunity against T. muris, both in chronic and acute models of infection, occurs via vaccination with adult-stage T. muris ES (69, 113). We chose to focus on identifying dominant T. muris antigens using protective Tm-ES antisera to immunoscreen the T. muris adult whole worm expressional cDNA library to select targets and express them as recombinant proteins for immunogenicity and efficacy testing in the AKR mouse model.
2.2 Materials and Methods

Preparation of *T. muris* excretory and secretory (ES) products and generation of immune sera

The *T. muris* ES products were obtained from the overnight culture of *T. muris* adult worms isolated from laboratory maintained STAT6/KO mice based on an established protocol (13, 69, 114). The protein concentration of Tm-ES was measured using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, USA) and determined to be endotoxin free (<0.88 EU/mg) by Charles River Endosafe®-PTS system (Charles River, USA). The concentrated *T. muris* ES products containing the stichosome-secreted proteins (100 µg/mouse) were formulated by emulsification with Montanide ISA 720 at 30:70 aqueous to oil based on volume, as described (103). The Tm-ES was administered to male AKR mice at 100 µg of antigen per dose in 100 µL, subcutaneously at the lateral tail base for a total of three immunizations at 2 week interval. Blood was collected from immunized mice 10 days after the last immunization by cardiac puncture. Serum was isolated from blood by centrifugation in serum-separating tube (Sarstedt, Germany) at 10,000 g for 5 minutes and stored at -80°C until assay.

Immunoscreening of the *T. muris* adult cDNA library

Mouse anti-ES sera were used to immunoscreen *T. muris* adult cDNA library to identify immunodominant antigens based on the methods described previously (115). Briefly, 5x10⁴ plaques of the cDNA library made from *T. muris* adult worm were plated on each LB agar plate. The expression of *T. muris* adult proteins was induced and transferred by covering each plate with a 10 mM IPTG soaked nitrocellulose membrane. The blotted membranes were incubated with 1:2,000 dilutions of mouse anti-ES sera and then reacted with HRP-conjugated goat anti-mouse IgG (1:5000) (Invitrogen, USA). The putative positive clones were scored and selected for secondary screening with the same reagents until a single positive clone was obtained.
The DNA sequences of positive clones were determined by double strand DNA sequencing using vector flanking primers, T3 and T7 RNA polymerase promoters (5′-TAATACGACTCACTATAGGG-3′ and 5′-ATTAAACCCTCCTAAAGGGA-3′, respectively) (Thermo Fisher Scientific, USA) and sequenced by Lone Star Labs (USA). Nucleotide and deduced amino acid sequences were compared to existing sequences by BLAST searching in the GenBank (http://www.ncbi.nlm.nih.gov) and in the *T. muris* reference genome on WormBase ParaSite (http://parasite.wormbase.org/Trichuris_muris_prjeb126/). MUSCLE 3.8.31 was used for multiple sequence alignment, PhyML 3.1 for phylogeny, and TreeDyn 198.3 for phylogenetic tree rendering using one click analysis mode online at http://www.phylogeny.fr (116, 117). The two most highly abundant proteins identified from this screening are *Tm*-WAP and *Tm*-CAP-1. These two proteins were expressed as recombinant proteins and purified as described below.

**Expression and purification of *Tm*-WAPs and *Tm*-CAP-1**

DNA encoding for the *rTm*-WAP49, comprising 70% of the immunodominant *Tm*-WAP (amino acids 24-393), was amplified without signal peptide from the total *T. muris* adult cDNA using forward (5′-GCGAATTTCGCTAAAATAGGTTCATGTCC-3′) and reverse (5′-ATCGGGCCCGCTCAATGATGATGATGATGCTCGCCAGTGCTGTCGTCTCGGT-3′) primers and subcloned into yeast expression vector pPICZαA (Invitrogen, USA) using the restriction enzyme sites of EcoRI and XbaI and confirmed by sequencing the vector flanking primers using α-factor and 3′AOX1, as previously described (118). The recombinant plasmids were linearized with SacI digestion and transformed into *Pichia pastoris* X33 by electroporation, according to the manufacturer’s recommendations (Invitrogen, USA). Transformed cells were plated on selective medium. Transformed colonies were then purified by streaking for isolated colonies on selective medium. The expression of recombinant *Tm*-WAP49 with 6His-tag at C-terminus was induced in medium with methanol in 10 L fermentation for 96 hours. The expressed recombinant *Tm*-WAP49 was purified with immobilized metal affinity chromatography (IMAC), as previously described (119).
For cloning *Tm*-WAP-F8+*Na*-GST-1, DNA coding for the *Tm*-WAP repeat fragment 8, the most conserved (69-96%) of the 10 repeats of *Tm*-WAP, was synthesized by GenScript. *Tm*-WAP-F8 was then fused with DNA coding for *Na*-GST-1 at N-terminus, a hookworm vaccine antigen with GST function that helps the solubilization of fusion protein (76, 108) and cloned into pET41a by using Ndel/Ndel/Xhol sites. The *Tm*-WAP-F8+*Na*-GST-1 was expressed as soluble recombinant fusion protein in *E. coli* BL21 under induction of 1 mM IPTG and purified with immobilized metal affinity chromatography.

For cloning *Tm*-CAP-1, the DNA encoding for *Tm*-CAP-1 (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein of *Trichuris muris*), without N-terminus signal peptide and C-terminus GPI anchor, was amplified from *T. muris* adult cDNA using forward (*Tm*-CAP-1-F2: GACATATGGCACCACTTACGGTCCCCAT) and reverse (*Tm*-CAP-1-R2: TCGCGGCGCTCAATGATGATGATGATGATGTTCTCTTTAGGAGAAGACGT) primers and subcloned into *E. coli* expression vector pET41A (Novagen, USA) using Nedl/Notl sites. The sequencing-confirmed correct recombinant plasmid was transformed into *E. coli* BL21 (DE3) and the recombinant *Tm*-CAP-1 protein (*rTm*-CAP-1) was expressed under 1mM IPTG induction at 30 °C overnight. The *rTm*-CAP-1 was expressed as insoluble inclusion body and solubilized in 8M urea. The *rTm*-CAP-1 with 8 his-tag at C-terminus was purified with IMAC. The purified urea-denatured *rTm*-CAP-1 was refolded in the refolding buffer (250 mM Arginine, 150mM NaCl, 50mM Tris, pH 8.0).
2.3 Results

Immunoscreening for the identification of highly abundant *T. muris* excreted-secreted (ES) antigens

Prior to the start of this dissertation, our lab found that parenteral immunization of *Tm*-ES formulated with Montanide ISA 720 adjuvant induced significant worm burden reduction (90%, \( p=0.0079 \)) in AKR mice, similar to previous efficacy studies using *Tm*-ES with complete Freund’s adjuvant (CFA) (72) or incomplete Freund adjuvant (IFA) (69). Protective antiserum from mice immunized with *Tm*-ES plus ISA 720 was used to screen the *T. muris* adult whole worm expressional cDNA library and identified a total of 102 positive clones. DNA sequencing revealed a whey acidic protein (*Tm*-WAP) as the most abundant of the candidates identified (63 clones). BLAST searching revealed that *Tm*-WAP shares 96.8% amino acid sequence identity with a gene product (TMUE_s0165000300) in the established *T. muris* genome on WormBase (120, 121). The *Tm*-WAP protein consists of several repeats (3-10) of a 50 amino acid fragment that share 69-96% inter-fragment sequence identity and possess six conserved cysteines predicted to form multiple disulfide bonds (*Figure 4A*) (DiANNA 1.1) (122–124). The *Tm*-WAP gene shares high homology, defined at greater than 40% sequence alignment (125), with the *T. trichiura* putative porin proteins, TT95 (21) and TT52 (23), at 54% and 47% amino acid sequence identity, respectively (*Figure 4B*).
Figure 4. Amino acid sequences and phylogenetic analyses of WAP protein from *T. muris*. (A) The predicted 547 amino acid sequence of *Tm*-WAP. The predicted signal peptide is highlighted in red (SignalP 4.1). The *Tm*-WAP protein contains ten repeats of a whey acidic protein (WAP)-type disulfide core domain that were aligned using CLUSTAL W and prepared for display using BOXSHADE. Identical amino acids are shaded in black and similar amino acids in gray. This 50 amino acid domain contains 6 conserved cysteine residues. The 49 kDa N-terminus was expressed in yeast as r*Tm*-WAP49 (in red box). The repeat fragment #8 was expressed in *E. coli* as *Tm*-WAP-F8 (in yellow box) using an Na-GST-1 expression tag. (B) Phylogenetic tree of *Tm*-WAP and its corresponding gene TMUE_s0165000300 to their respective homologues in different *Trichuris* species.
The second most abundant clone identified from the cDNA library screening was *T. muris* Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 (CAP-1) protein (35 clones) (Figure 5A). The CAP-domain family (also known as SCP/TAPS) was previously identified based on its abundance in the secretome of whipworm and other soil-transmitted helminths (126, 127). BLAST searching revealed that *Tm*-CAP-1 shares 99.3% amino acid sequence identity with a gene product (TMUE_s0030008500) in the established *T. muris* genome on WormBase (120, 121). *Tm*-CAP-1 shares the highest homology with the *T. suis* SCP-like protein (accession number KHJ42268.1) and *T. trichiura* CAP-domain containing protein (accession number CDW52210.1), at 48% and 38% amino acid sequence identity, respectively (Figure 5B).

A

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1  MFAAVLLAVCSLATLAA
19  APLTVPIMENDRSKIIISTVNKYASLQAANMEALTEWTDELARAQDIGA
69  KCEVTATDDTYGAGSLLDVTQVITVDTLADALKTEEAYTVGTDTCNASA
119  IDKCNGYKQFVWYKGKGGCAITICDANGPNGGNTFTCVFEHKATLDPS
169  QKPYVGKTACTFCSSSWSTCNSLCTSSNVLAPTSCGSKPSKLTPLHRF
219  VYKATSATILSQSQTITDSTIAPIFQYQGIFYVSSSEDACGQLRPMK
269  LSAVGKIDFVYVGEELFSYNNQNNYVETLGYVVPNAGFCEANATAHQ
319  FIRAAAAYNYTADVLLLREGANAVYFWDNAVFSFWSSA  364
```
The near full-length Tm-WAP was expressed with a 6His-tag at C-terminus in Pichia pastoris X33 as a soluble protein and purified by IMAC. After multiple unsuccessful attempts at expressing the entire protein in E. coli, ultimately yeast was found to be an efficient expression platform. The recombinant protein migrated at 49 kDa, therefore named rTm-WAP49. The most conserved repeat of Tm-WAP, fragment 8, was expressed with a Necator americanus glutathione s-transferase-1 (Na-GST-1)-tag at the N-terminus as a soluble recombinant fusion protein in E. coli BL21 under induction of 1 mM IPTG and purified with immobilized IMAC. In addition to improving the expression and solubility of the fusion protein, Na-GST-1 is a hookworm vaccine candidate (108), and selected for the potential as a future pan-anthelminthic vaccine candidate. The full-length recombinant Tm-CAP-1 protein was expressed in Escherichia coli (E. coli) BL21 as an insoluble inclusion body and solubilized in 8M urea. The rTm-CAP-1 with an 8 his-tag at the C-terminus was purified with immobilized metal affinity chromatography (IMAC), as previously described (119).

Figure 5. Amino acid sequences and phylogenetic analyses of CAP-1 protein from T. muris. (A) The predicted 361 amino acid sequence of Tm-CAP-1. The predicted signal peptide is highlighted in red (SignalP 4.1). (B) Phylogenetic tree and sequence identify of rTm-CAP-1 and corresponding gene TMUE (s0030008500), to their respective homologues in different Trichuris species.
2.5 Discussion

With nearly a half-billion people afflicted, trichuriasis is one of the most prevalent infectious diseases in the world, and yet there are limited prophylactic and therapeutic options. A vaccine against human whipworm would provide a low-cost and long-term solution, yet the identification of candidates is lacking. Here we report the identification of two highly abundant secretory proteins, whey acidic protein (WAP) and Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 (CAP-1). Immunoscreening of the adult *T. muris* expressional cDNA library with protective *Tm*-ES antisera identified *Tm*-WAP, followed by *Tm*-CAP-1 as the most recognized proteins. The high degree of amino acid sequence identity for the CAP and WAP proteins between *T. muris* and *T. trichiura* also suggests that key immunological epitopes may be shared between the macromolecules.

Validating our results identifying *Tm*-WAP as the dominant antigen from among the *T. muris* ES proteins, a recent report by Shears et al. (2018) identified that TMUE_s0165000300 (*Tm*-WAP gene) amongst the most abundant proteins within *T. muris* ES (113). In these studies, Shears et al. divided *T. muris* ES into four sub-fractions using size exclusion chromatography, to search for protective immunogens on sub-fractions that induced protective immunity following immunization. *Tm*-WAP was recognized as a major component of two potent ES preparations, sub-fractions 3 and 4, which induced sterile (or near sterile) immunity against a subsequent low dose *T. muris* infection (113). The authors further described sub-fractions 3 and 4 as potent stimulants of IL-13 in infection-primed lymphocytes, likely contributing to their efficacy. Given that *Tm*-WAP is highly abundant within sub-groups 3 and 4, it is possible that it is one of the key protective components within these fractions. TMUE_s0030008500 (*Tm*-CAP-1 gene), however, was not reported among the *Tm*-ES fractions.
Chapter III

Evaluate the immunogenicity and efficacy of *T. muris* recombinant proteins in a murine model
3.1 Introduction:

The overarching goal of this research is to identify protective candidate antigens for the eventual development as a vaccine against trichuriasis. As described in Chapter 1, we identified two secretory proteins, Tm-WAP and Tm-CAP-1, by an immunoscreen of the T. muris cDNA expressional library using protective Tm-ES antisera. In this chapter we determine the induction of humoral and cellular immune responses by recombinant Tm-WAP and Tm-CAP-1 proteins and, subsequently, whether immunization provide efficacy against T. muris challenge.

The closely related Trichuris muris parasite, specific to mice, is a well-established surrogate model for human T. trichiura disease pathophysiology and subsequent host immunity, used for evaluating the immunogenicity and efficacy of vaccine candidates (13, 14). In both mice and humans, ingested whipworm eggs hatch into larvae in the terminal ileum and caecum due to interactions with the host microbiome (16). There, the helminths’ stichosome is thought to secrete products (i.e. Tm-ES) that facilitate essential pore-formation in the host colonic mucosa and host immunomodulation (14, 22). Once embedded, the Trichuris worms become fixed in location, causing inflammation at the site of intracellular attachment and induce colitis-like pathology (14). We and others have reported that immunization with T. muris ES product elicits protective immunity in murine models (69, 75, 128).

Protective immunity in preclinical models correlates with T helper type 2 (TH2) responses, with production of cytokines IL-4, IL-9, and IL-13 along with IgG1 antibodies and high IgG1 to IgG2a ratios (14, 69, 75, 114, 129, 130). Conversely, susceptibility is characterized by a predominate TH1 response (IL-12 and Interferon [IFN]-γ) and IgG2a antibodies (14, 69, 129).

Here we report that recombinant Tm-WAP (rTm-WAP49) induced significant protection against T. muris challenge, while recombinant Tm-CAP-1 was ineffective. Protection against T. muris infection by rTm-WAP49 was associated with strong TH2 cellular and humoral immune responses.
3.2 Materials and Methods

Ethics statement

All animal procedures were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC - AN-6297) in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals.

Mice

6-7-week-old male AKR mice were purchased from Jackson Labs (USA) and used at 5 mice per group for immunogenicity analyses and 13-15 mice per group for efficacy studies. Mice for the challenge studies were infected by oral gavage of 300 embryonated eggs in 100 ul of diH2O. Worm burden and post-challenge immunological response were assessed at day 15 post-infection. Blood collection at various time points was by tail bleeding and the terminal bleeds were collected by cardiac puncture at the time of sacrifice. The spleens, mesenteric lymph nodes, and inguinal lymph nodes were collected at the time of sacrifice, and immediately resuspended in complete RPMI media (RPMI 1640 with L-glutamine [VWR, USA], 10% heat inactivated FBS [VWR, Radnor, PA], and 1x pen/strep solution [Thermo Fisher Scientific, USA]) for immunological analysis.

Immunization

The protein concentration of each antigen (rTm-WAP49; rTm-WAP-F8+Na-GST-1; rTm-CAP-1; Tm-ES) was measured using BCA (Thermo Fisher Scientific, USA) and each of the recombinant protein was also ascertained to be endotoxin free (<0.88 EU/ mg) by Charles River Endosafe®-PTS system (Charles River, USA). All immunogens were prepared in sterile, ultrapure grade phosphate-buffered saline (PBS) (VWR, USA) and emulsified with Montanide
ISA 720 at 30:70 aqueous to oil based on volume, as described (103). The Tm-ES as well as rTm-WAP49, rTm-WAP-F8+Na-GST-1, and rTm-CAP-1 were administered at 100 µg in 100 µL per dose, subcutaneously at the lateral tail base.

**T. muris parasite challenge**

*T. muris* E (Edinburgh) isolate, kindly provided by Dr. Joe Urban from the USDA, were maintained in susceptible STAT6K/O mice. At day 42 post infection (p.i.), adult worms were gently extracted by forceps from the caecum of sacrificed mice. *T. muris* eggs collected from cultured *T. muris* adult worms were washed in sterile water and centrifuged at 500xg for 5 minutes and supernatant debris was removed from pelleted eggs. Collected eggs were stored in distilled water in culture flasks (VWR, USA) and allowed to embryonate at room temperature in the dark for 8 weeks. 300 embryonated eggs were separated into 100 µL aliquots. Each aliquot was counted by light microscopy to verify 300 ± 10 embryonated eggs.

**Antibody analysis**

Sera were isolated from blood by centrifugation in serum-separating tube (Sarstedt, Germany) at 10,000 g for 5 minutes and stored at -80°C until assay. Samples were evaluated for antigen-specific immunoglobulins IgG (Lifespan Biosciences, USA), IgG1 (Lifespan Biosciences, USA), IgG2a (Lifespan Biosciences, USA), and IgE (SouthernBiotech, USA) by a modified indirect enzyme-linked immunosorbent assay (ELISA), as described previously (131). Concentrations of rTm-WAP49 (0.375 µg/mL), rTm-WAP-F8+Na-GST-1 (0.375 µg/mL), rTm-CAP-1 (0.375 µg/mL), rNa-GST-1 (0.375 µg/mL), or Tm-ES (0.75 µg/mL) used for ELISA were selected based on pretested optimal signal/noise ratio. The absorbance was measured by dual wavelength analysis at test wavelength of 450 nm and the reference wavelength of 630 nm using a spectrophotometer (BioTek, USA).

**Cytokine analysis**
Spleens, mesenteric lymph nodes (MLNs), and inguinal lymph nodes (ILNs) were obtained from mice two weeks after the third immunization (immunogenicity groups) or 15 days after challenge infection (efficacy groups). We identified the ILNs as the primary site of vaccine drainage when administered subcutaneously at the lateral tail base by tracking 5% Evan's Blue ink drainage 30 minutes after administration, as described by Harrell et al. (132). Tissues were disassociated into a single-cell suspensions using a 100 μm cell strainer and resuspended in complete RPMI medium. After a 5-minute centrifugation (300 x g), splenocytes were resuspended in 2 mL of ACK lysis buffer (Thermo Fisher Scientific, USA) and incubated at room temperature for 5 minutes to remove red blood cells. After centrifugation cells were resuspended in complete RPMI media and incubated for 2 hours in a 37°C + 5% CO₂ incubator before counting the number of live cells using a Cellometer Auto 2000 Cell Viability Counter (Nexelcom, USA). To have sufficient cells for multiple cytokine assays, MLNs and ILNs, were separately pooled into 3 sets of 5 mice per tissue.

Cells were seeded in a 96-well U-bottom culture plate (Corning, USA) at 1x10⁶ cells per well in 250 μl medium and restimulated with either \( rTm\)-WAP49 (10 µg/mL), \( rTm\)-WAP-F8+Na-GST-1 (10 µg/mL), \( rTm\)-CAP-1 (10 µg/mL), \( rNa\)-GST-1 (10 µg/mL), or \( Tm\)-ES (50 µg/mL) at 37°C, 5% CO₂ for 72 hours. An unstimulated, media only negative control and a positive control where cells were activated with a mixture of 20 ng/mL PMA and 1 μg/mL Ionomycin were included in all the assays. After 72 hours the cells were pelleted by centrifugation at 300 x g for 5 min and the supernatants were collected for measuring the amount of cytokine produced. Concentrations of different stimulants and duration of stimulation were selected based on optimal pretested antigen to negative control ratio. Initially supernatant samples were tested using a custom Bio-Plex Pro Mouse Cytokine 10-plex kit (Bio-Rad, USA) for determining the levels of IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p70), IL-13, IL-17A, IFN-γ and TNF-α. To evaluate accessory cytokines/chemokines associated with protection, subsequent experiments used the expanded Bio-Plex Pro Mouse Cytokine 23-plex kit, which included the original 10 cytokines along with
Eotaxin, G-CSF, GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-12 (p40), KC, MCP-1 (MCAF), MIP-1α, MIP-1β, and Rantes. To increase the sensitivity of the experiment, the kit was used in combination with DA-Bead plates (Curiox Biosystems, Singapore), as previously described (133). Samples were run on a Luminex® Magpix® multiplex reader according to manufacturer's recommendations (Luminex, USA) and analyzed using the Bio-Plex Manager 6.0 software (Bio-Rad, USA). Sensitivity cutoff values for the detection of different cytokines were dependent on the included standards for each lot number of the Bio-Rad Bio-Plex kit. Cytokine values from supernatants of cells cultured with media only were deemed background and thus were subtracted from the values of antigen stimulated samples.

**Electrophoresis and immunoblotting of recombinant Tm-WAP proteins**

Samples were subjected to electrophoresis using a 4-20% gradient NuPAGE/MES gel (Thermo Fisher Scientific, USA) and either immediately stained with Coomassie Blue for imaging analysis or transferred onto PVDF membrane (Thermo Fisher Scientific, USA). Subsequently, the PVDF membranes were blocked with 5% (w/v) skim milk powder in PBST (PBS +0.05% Tween-20), and then incubated with sera (1:3,000) from immunized mice (r Tm-WAP49, r Tm-WAP-F8+Na-GST-1 or Tm-ES). HRP-conjugated goat anti-mouse IgG (Invitrogen, USA) was used as a secondary antibody, followed by ECL substrate (GE Healthcare, USA) to develop antibody bound bands. The nitrocellulose membranes of anti-r Tm-WAP-F8+Na-GST-1 and anti-Tm-ES were cut just prior to ECL substrate reaction between lanes 4-5 and 3-4, respectively, to allow for different length of reaction. Both T. muris adult homogenate and Tm-ES were used at 10 µg for SDS-PAGE and 2.0 µg for western blot analyses. The r Tm-WAP49 protein was used at 2 µg for SDS-PAGE and 200 ng for western blot. Both r Tm-WAP-F8+Na-GST-1 and r Na-GST-1 were used at 2 µg for SDS-PAGE and 100 ng for western blot.

**Data presentation and statistical analyses**
Figures 6-16 were generated in GraphPad 7.04, with the exception of Figure 7 A-C, Figure 10 A-C, and Figure 14 B, D, and F, and Figure 15 B. To compare groups based on data from unknown normality of distribution and small sample size (n < 20 per group), we performed non-parametric analyses employing Kruskal-Wallis H test with Dunn’s Multiple Comparisons and Mann-Whitney U test using GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com).

Figure 7 A-C, Figure 10 A-C, and Figure 14 B, D, and F, data was loaded into R programming language environment version 3.4.3 using Rstudio version 1.1.423 (http://www.rstudio.com/). Readxl package was used to import the data from the spreadsheet (https://CRAN.R-project.org/package=readxl). Tidyverse package (version 1.2.1) was used to transform the imported data for the analysis (https://CRAN.R-project.org/package=tidyverse). Mean value for each cytokine in a group was calculated by bootstrapping using smean.cl.boot function with the default setting of Hmisc package (version 4.1-1) (https://CRAN.R-project.org/package=Hmisc). We used Wilcoxon test for comparing values of each cytokine between groups using coin package (version 1.2-2) and the effect size was calculated using Cliff delta using effsize package (134, 135). A plot of the calculated results was generated using ggplot2 package (version ggplot2 2.2.1) (136).

For all graphs, statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001. Effect size: d<0.2, d<0.5, d<0.8, and d>0.8, correspond to N (negligible), S (small), M (medium), and L (large), respectively.
3.3 Results

Immunogenicity and protective efficacy of recombinant *Tm*-WAP and *Tm*-CAP-1 proteins

As described in chapter II above *Tm*-CAP-1 protein expression in *E. coli* was successful, but expression of *Tm*-WAP necessitated changing the vector system from *E. coli* to yeast. Therefore, while the expression of *Tm*-WAP and the purification of the recombinant protein were in progress, we expressed the most conserved repeat of *Tm*-WAP, fragment 8, with a *Necator americanus* glutathione s-transferase-1 (Na-GST-1)-tag at the N-terminus and successfully produced a soluble recombinant fusion protein in *E. coli*. The resulting recombinant protein, r*Tm*-WAP-F8+Na-GST-1 was tested for immunogenicity along with recombinant *Tm*-CAP-1 protein in the AKR mouse model.

Mice were immunized three times at two-week intervals with *Tm*-ES, r*Tm*-WAP-F8+Na-GST-1 or r*Tm*-CAP-1 using the Montanide ISA 720 adjuvant (Figure 12A). Montanide ISA 720 is a squalene-based water-in-oil emulsion used in multiple efficacious helminth preclinical animal model studies, including the closely related *Trichinella spiralis*, with safety in humans (102–104). Control groups included mice injected with PBS or adjuvant alone. Two weeks after the final immunization, serum levels of antigen-specific IgG1 and IgG2a were determined by ELISA to calculate end-point titers. Mice immunized with *Tm*-ES, r*Tm*-WAP-F8+Na-GST-1, or r*Tm*-CAP-1 exhibited significant levels of respective antigen-specific IgG1 as well as IgG2a titers relative to those in control groups of mice immunized with PBS or adjuvant ISA-720 (Figure 6A and 6B). However, antigen-specific IgG2a levels were significantly higher in mice immunized with r*Tm*-CAP-1 relative to those in mice immunized with *Tm*-ES (Figure 6C). Consequently, mice immunized with r*Tm*-CAP-1 had a significantly lower IgG1:IgG2a ratio relative to those immunized with either *Tm*-ES or r*Tm*-WAP-F8+Na-GST-1 (Figure 6D).
Figure 6. Humoral immune responses induced by immunization with \( rTm\)-WAP-F8+Na-GST-1 and \( rTm\)-CAP-1 proteins in the AKR murine model. (A) Scheme for immunization and sample collection. Humoral immune response was measured by determining the endpoint serum titer for antigen-specific (B) IgG1 and (C) IgG2a by ELISA to calculate the (D) IgG1 to IgG2a ratio for the mice in each group. Mice in groups injected with PBS or Montanide ISA 720 adjuvant were used as negative controls. Statistical significance: *\( p<0.05\), **\( p<0.01\), ***\( p<0.001\), ****\( p<0.0001\).
Next, an analysis of antigen-specific T cell response of mice immunized with the different antigens was conducted. Spleen cells were isolated and restimulated with the corresponding candidate proteins for 72 hours in vitro, with Tm-ES serving as a positive control. In comparison to the mice in the two negative control groups (PBS or adjuvant control) antigen-specific T cell responses in mice immunized with Tm-ES (Figure 7A), rTm-WAP-F8+Na-GST-1 (Figure 7B), and rTm-CAP-1 (Figure 7C) were significantly increased. While there was a significant difference in IFN-γ production between mice immunized with rTm-WAP-F8+Na-GST-1 compared to rTm-CAP-1 when stimulated with cognate antigen (p=0.003) (Table 3), there was no difference in IFN-γ production between mice immunized with rTm-WAP-F8+Na-GST-1 compared to control mice immunized with ISA 720 adjuvant when stimulated with rTm-WAP-F8+Na-GST-1 (p>0.999) (Figure 7B). On the other hand, production of TH2 cytokines IL-4 and was significantly higher in mice immunized with either rTm-WAP-F8+Na-GST-1 (p=0.011) or Tm-ES (p=0.048) compared to those immunized with rTm-CAP-1 when stimulated with cognate antigen (Table 3). Cumulatively, there was a larger TH2 response in mice immunized rTm-WAP-F8+Na-GST-1 and Tm-ES compared to those immunized with rTm-CAP-1 (Figure 7).
B

**Stimulant:** rTm/WAP-F8+Na -GST

**Immunization:**
- ISA 720
- rTm-WAP-F8+Na-GST-1 + ISA 720

**Effect Size:**
- L - Large
- M - Medium
- S - Small
- N - Negligible

**P-value:**
- * < 0.05
- ** < 0.01
- *** < 0.001
- **** < 0.0001

C

**Stimulant:** rTm/CAP-1

**Immunization:**
- ISA 720
- rTm-CAP-1 + ISA 720

**Effect Size:**
- L - Large
- M - Medium
- S - Small
- N - Negligible

**P-value:**
- * < 0.05
- ** < 0.01
- *** < 0.001
- **** < 0.0001
Figure 7. Cellular immune responses in terms of cytokine levels in the supernatants of splenocytes stimulated with media or cognate antigens. Splenocytes from mice in each group were stimulated with media or cognate antigen, (A) Tm-ES, (B) rTm-WAP-F8+Na-GST-1, or (C) rTm-CAP-1 for 72 hours and the cytokines were detected using a 10-plex assay kit. Mice in groups injected with Montanide ISA 720 were used as negative controls. Cells receiving no stimulus (media only) were subtracted as background from stimulated cells from corresponding mice. Values from duplicate wells for each treatment/stimulation were averaged for individual cytokines for the 5 mice in each group to calculate statistical significance. Data are presented as means ± 95% confidence interval. Statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001. Effect size: d<0.2, d<0.5, d<0.8, and d>0.8, correspond to N (negligible), S (small), M (medium), and L (large), respectively. (D) Levels of TH1 and TH2 cytokines produced in response to stimulation with cognate antigens are shown as stacked columns.

Table 3. Analyses of TH1 and TH2 cytokine levels in the supernatants of cells stimulated with Tm-ES, rTm-WAP-F8+Na-GST-1, or rTm-CAP-1. Cytokine levels of ES (Tm-ES), WAP-F8 (rTm-WAP-F8+Na-GST-1), and CAP (rTm-CAP-1) were compared by Kruskal-Wallis H test with Dunn’s Multiple Comparisons. Data are presented as mean. Statistical significance: not significant (NS)>0.05, *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.
To evaluate whether the pronounced TH2 immune responses induced in mice immunized with the different antigens were effective in controlling *T. muris* infection, mice immunized with *rTm*-WAP-F8+Na-GST-1, *rTm*-CAP-1, *Tm*-ES, or Montanide ISA 720 adjuvant alone were challenged orally with 300 embryonated *T. muris* eggs two weeks after the final immunization. The intestinal worm burden was assessed 15 days post-infection by direct microscopy (Figure 8A). In agreement with data from our earlier reported studies and those of others (69, 113), mice immunization with the positive control *Tm*-ES exhibited a significant reduction (93%, p<0.0001) in mean worm burden compared to mice immunized with Montanide ISA 720 adjuvant alone (Figure 8B). Furthermore, immunization with *rTm*-WAP-F8+Na-GST-1 also induced significant protection (27% reduction in worm burden, p=0.0124), while mice immunized with *rTm*-CAP-1 showed only 3% reduction in worm burden (p=0.845).

![Figure 8](image.png)

**Figure 8.** Protective efficacy of *rTm*-WAP-F8+Na-GST-1 and *rTm*-CAP-1 proteins. (A) Immunization and challenge schedule. (B) Efficacy in terms of reduction in worm burden in the different groups of immunized mice was evaluated on day 15 post-infection by counting the numbers of worms in the intestines by microscopy.
In addition to strong humoral and cellular TH2 response generated by vaccination, a continued TH2 immune response is necessary during the “critical period” of worm expulsion (day 0-21 p.i.) (69). To evaluate the humoral response post challenge, we measured the antigen-specific IgG1 (Figure 9A) and IgG2a (Figure 9B) levels in the serum along with T cell responses in splenocytes after restimulation in vitro with the cognate antigens to the initial immunization. In mice protected by immunization with *Tm*-ES or *rTm*-WAP-F8+Na-GST-1, relative to adjuvant control mice, we observed a significant increase in TH2 immune responses, characterized by high IgG1:IgG2a ratios (Figure 9C). Antigen-specific IgG1:IgG2a ratios were significantly lower in mice immunized with non-protective *rTm*-CAP-1 compared to protective *Tm*-ES and *rTm*-WAP-F8+Na-GST-1.

Figure 9. Post-challenge humoral immune response in mice immunized with *rTm*-WAP-F8+Na-GST-1 and *rTm*-CAP-1 proteins. End-point serum titers for antigen-specific (A) IgG1 and (B) IgG2a were determined by ELISA to calculate the (C) IgG1 to IgG2a ratio for the mice in each group. Mice in groups injected with Montanide ISA 720 were used as negative controls. Statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.
Post-challenge cellular response was measured in the supernatant of splenocytes stimulated with cognate antigens *Tm*-ES (Figure 10A), *rTm*-WAP-F8-Na-GST-1 (Figure 10B) and *rTm*-CAP-1 (Figure 10C). As with pre-challenge, mice immunized *Tm*-ES, *rTm*-WAP-F8-Na-GST-1 or *rTm*-CAP-1 had no significant difference in production in TH1 cytokines IL-12 or IFN-γ in splenocytes stimulated with cognate antigen (Table 4). However, the levels of TH2 cytokines IL-4, IL-5, and IL-13 were significantly higher for all three antigen groups compared to adjuvant alone (Figure 10A-C). Production of TH2 cytokine, IL-9, which is important for worm expulsion through enhancement of gut peristalsis and tissue mast cell maturation (88, 89), was only elevated in mice immunized with *Tm*-ES (p=0.007) and nearly so for *rTm*-WAP-F8-Na-GST-1 (p=0.054), compared to mice immunized with *rTm*-CAP-1 (Table 4). Furthermore, mice immunized with *rTm*-CAP-1 had significantly lower levels of TH2 cytokines, namely IL-4 (p<0.0001), IL-5 (p=0.006), and IL-13 (p<0.0001) when compared to those in mice immunized with *rTm*-WAP-F8+Na-GST-1 (Table 4). A similar result was seen between mice immunized with *Tm*-ES compared to *rTm*-CAP-1 (Table 4). The cumulative TH2 cytokine level between *rTm*-WAP-F8+Na-GST-1 and *Tm*-ES was comparable (Figure 10D).
B

Stimulant: rTm-WAP-F8+-Na-GST-1

Immunization:
- ISA 720
- rTm-WAP-F8+-Na-GST-1 + ISA 720

Effect Size:
- L - Large
- M - Medium
- S - Small
- N - Negligible

P-value:
- * < 0.05
- ** < 0.01
- *** < 0.001
- **** < 0.0001

C

Stimulant: rTm-CAP-1

Immunization:
- ISA 720
- rTm-CAP-1 + ISA 720

Effect Size:
- L - Large
- M - Medium
- S - Small
- N - Negligible

P-value:
- * < 0.05
- ** < 0.01
- *** < 0.001
- **** < 0.0001
Figure 10. Post-challenge cellular immune responses in terms of cytokine levels in the supernatants of splenocytes stimulated with media or cognate antigens. Splenocytes from mice in each group were stimulated with media or cognate antigen, (A) Tm-ES, (B) rTm-WAP-F8+Na-GST-1, or (C) rTm-CAP-1 for 72 hours and the cytokines produced were detected using a 10-plex assay kit. Values for the levels of cytokines from cells receiving no stimulus (media only) were subtracted as background from those in cells stimulated with the cognate protein in the corresponding mice. Values from duplicate wells for each treatment/stimulation were averaged for individual cytokines for the 13-15 mice in each group to calculate statistical significance. Mice in groups injected with Montanide ISA 720 were used as negative controls. Data are presented as means ± 95% confidence interval. Statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001. Effect size: d<0.2, d<0.5, d<0.8, and d>0.8, correspond to N (negligible), S (small), M (medium), and L (large), respectively. (D) Levels of cytokines for each group are shown as stacked columns based on TH1 vs TH2 classification for each cognate antigen.

Table 4. Analyses of post-challenge TH1 and TH2 cytokine levels in the supernatants of cells stimulated with Tm-ES, rTm-WAP-F8+Na-GST-1, or rTm-CAP-1. Cytokine levels of ES (Tm-ES), WAP-F8 (rTm-WAP-F8+Na-GST-1), and CAP (rTm-CAP-1) were compared by Kruskal-Wallis H test with Dunn’s Multiple Comparisons. Data are presented as mean. Statistical significance: not significant (NS)>0.05, *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.
Recombinant WAP protein (rTm-WAP49) induces strong type 2 protective immunity

After initial unsuccessful attempts to express Tm-WAP in *E. coli*, rTm-WAP49 comprising of nearly eight of the ten, 50 amino acid repeats that make-up the full-length Tm-WAP protein was expressed in *Pichia pastoris* X33. We evaluated the immunogenicity and protective efficacy of rTm-WAP49 in comparison to that of rTm-WAP-F8+Na-GST-1 following the immunization and challenge schedule shown in Figure 8A. Since the WAP fragment 8 was expressed and used with the Na-GST-1 tag for the immunization and challenge studies described above, and because Na-GST-1 corresponds to a hookworm candidate vaccine antigen, we decided to verify the contribution of the Na-GST-1 tag within the rTm-WAP-F8+Na-GST-1 for the observed protective efficacy against *T. muris*. Therefore, the recombinant Na-GST-1 protein was included as an additional control immunogen. Montanide ISA 720 adjuvant alone and Tm-ES were used as negative and positive control reagents, respectively. Endpoint titers of antigen-specific IgG1 (Figure 11A) and IgG2a (Figure 11B) in the serum samples were significantly higher for all immunization groups compared to adjuvant alone. While the cognate antigen-specific IgG1:IgG2a ratios in mice immunized with rTm-WAP-F8+Na-GST-1 or rTm-WAP49 were significantly higher compared to the adjuvant alone, the ratios were lower in mice immunized with rTm-WAP-F8+Na-GST-1, relative to that in mice immunized with Tm-ES. However, the IgG1:IgG2a ratios in mice immunized with rTm-WAP49 were comparable to that in mice immunized with Tm-ES (Figure 11C).
Next, we compared the efficacy and immune correlates for $r_{Tm}$-WAP-F8+$Na$-GST-1 and $r_{Na}$-GST-1 as immunogens using the same immunization and challenge schedule as described above. We also included a group of mice immunized with $r_{Na}$-GST-1 to account for its potential contribution to protection observed for $r_{Tm}$-WAP-F8+$Na$-GST-1. In comparison to the Montanide ISA 720 adjuvant control, a significant reduction in worm burden was observed in mice immunized with $r_{Tm}$-WAP49 (48%, $p=0.000103$) or $r_{Tm}$-WAP-F8+$Na$-GST-1 (38%, $p=0.00278$), as well as the positive control $Tm$-ES (89%, $p<0.0001$), with no significant difference between

![Image](image-url)
rTm-WAP-F8+Na-GST-1 and rTm-WAP49. Importantly, immunization with rNa-GST-1 did not lead to a significant reduction in worm burden after challenge suggesting that the protective efficacy for the rTm-WAP-F8+Na-GST-1 was related to the WAP fragment 8, and not the Na-GST-1 tag (Figure 12).

Immune correlates for the protective efficacy by rTm-WAP49 and rTm-WAP-F8+Na-GST-1 included significantly higher antigen-specific IgG1 endpoint titers post-infection compared to that in mice immunized with the Montanide ISA 720 adjuvant alone (Figure 13A). However, rTm-WAP-F8+Na-GST-1 generated a higher antigen-specific IgG2a response (Figure 13B), leading to a significantly lower IgG1:IgG2a ratio relative to that observed in mice immunized with rTm-WAP49 (Figure 13C).
We also analyzed post-challenge cellular immune correlates of protection in the spleen, the parasite-draining mesenteric lymph nodes (MLNs), and the vaccine-draining inguinal lymph nodes (ILNs). In mice immunized with rTm-WAP-F8+Na-GST-1 or rTm-WAP49, stimulation of splenocytes with cognate antigen showed high levels of TH2 cytokines IL-4, IL-5, IL-6, IL-9 and IL-13 (Figure 14A). In comparing the two WAP derivative TH2 cytokine levels, IL-4, IL-5, and IL-6 were all produced into the cellular supernatant at similar levels to cognate antigen stimulation of splenocytes (Table 5A). Interestingly, we observed that the IL-13 response was significantly

Figure 13. Post-challenge humoral immune responses in mice immunized with rTm-WAP49 or rTm-WAP-F8+Na-GST-1. At the time of sacrifice, humoral immune response in terms of end-point serum titers for antigen-specific (A) IgG1 and (B) IgG2a were measured by ELISA to calculate (C) IgG1 to IgG2a ratio. Mice in groups injected with Montanide ISA 720 were used as negative controls. Statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.
higher for rTm-WAP-F8+Na-GST-1 (p=0.01) whereas the IL-9 response was significantly higher for rTm-WAP49 (p<0.0001), relative to each other in the splenocytes (Figure 14B) (Table 5A). A significant TH2 response was observed in the MLNs of mice immunized with rTm-WAP-F8+Na-GST-1 or rTm-WAP49 (Figure 14C) compared to mice immunized with ISA 720 adjuvant and stimulated with either rTm-WAP-F8+Na-GST-1 or rTm-WAP49, respectively. However, there was no significant difference in TH1 or TH2 cytokine levels in the cellular supernatants of MLNs stimulated by rTm-WAP-F8+Na-GST-1 or rTm-WAP49 (Figure 14D) (Table 5B). A similar, significant TH2 responses was seen in the ILNs of mice immunized with rTm-WAP-F8+Na-GST-1 or rTm-WAP49 compared to mice immunized with ISA 720 (Figure 14C). Again, there was no observed difference in TH1 or TH2 cytokine levels of ILNs stimulated by rTm-WAP-F8+Na-GST-1 or rTm-WAP49 (Figure 14F) (Table 5C).
A

Stimulant: rTm-WAP49

Stimulant: rTm-WAP-F8+Na-GST-1

Cytokines

IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-12(p40)
IL-12(p70)
IL-15
IL-17A
IFN-γ
Eotaxin
GM-CSF
KC
MCP-1
MIP-1α
MIP-1β
RANTES
TNF-α

Cytokine concentration (pg/ml)

B

Splenocytes

TH1

TH2

Immunization:

1. ISA 720
2. rTm-WAP49 + ISA 720
3. rTm-WAP-F8+Na-GST-1 + ISA 720

Effect Size:

L - Large
M - Medium
S - Small
N - Negligible

P-value:

* < 0.05
** < 0.01
*** < 0.001
**** < 0.0001

Stimulant:

A. rTm-WAP49
B. rTm-WAP-F8+Na-GST-1

Concentration (pg/mL)

Vaccine: 1 2 3
Stimulant: A B
C

Mesenteric Lymph Nodes

Stimulant: rTm -WAP49

Stimulant: rTm -WAP-F8+Na -GST-1

Cytokines

IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-10
IL-12(p40)
IL-12(p70)
IL-13
IL-17A
IFN-γ
Eotaxin
G-CSF
KC
MCP-1
MP-13
MP-16
RANTES
TNF-α

Cytokine concentration (pg/ml)

Effect Size:
L - Large
M - Medium
S - Small
N - Negligible
P-value:
* < 0.05
** < 0.01
*** < 0.001
**** < 0.0001

D

Mesenteric Lymph Nodes

TH1

TH2

Immunization

TH1

TH2

Concentration (pg/mL)

Vaccine: 1 2 3

Stimulant:

A rTm-WAP49
B rTm-WAP-F8+Na-GST-1

Stimulant:

IFN-γ
IL-12
IL-4
IL-5
IL-6
IL-9
IL-13

A 1 ISA 720
2 rTm-WAP49 + ISA 720
3 rTm-WAP-F8+Na-GST-1 + ISA 720
Figure 14. Post-challenge cellular immune responses in terms of cytokine levels in the supernatants of cells stimulated with media or cognate antigens. Cytokines detected in the supernatants of (A-B) splenocytes, (C-D) mesenteric lymph nodes (MLNs), or (E-F) inguinal lymph nodes (ILNs) after stimulation with media only or cognate antigen, \( rTm-WAP-F8+Na-GST-1 \) (10 μg/mL) or \( rTm-WAP49 \) (10 μg/mL) for 72 hours were detected using a 23-plex assay kit. Cytokines stacked based on TH1 vs TH2 group for each cognate antigen and compared to ISA 720 controls stimulated with the corresponding antigen. Mice in groups injected with Montanide ISA 720 were used as negative controls. Cells receiving no stimulus (media only) were subtracted as background from stimulated cells from corresponding mice. Values from duplicate wells for each treatment/stimulation were averaged for individual cytokines for the 15 mice in each group to calculate statistical significance. Data are presented as means ± 95% confidence interval. Statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001. Effect size: d<0.2, d<0.5, d<0.8, and d>0.8, correspond to N (negligible), S (small), M (medium), and L (large), respectively.
### Table 5. Analyses of post-challenge TH1 and TH2 cytokine levels in the supernatants of cells stimulated with \( rTm\)-WAP49 or \( rTm\)-WAP-F8+Na-GST-1.

Cytokine levels of WAP49 (\( rTm\)-WAP49) and WAP-F8 (\( rTm\)-WAP-F8+Na-GST-1) in (A) splenocytes, (B) mesenteric lymph nodes, and (C) inguinal lymph nodes, were compared by Kruskal-Wallis H test with Dunn’s Multiple Comparisons. Data are presented as mean. Statistical significance:

**Statistical significance:**
- NS: not significant (\( p > 0.05 \))
- *: \( p < 0.05 \)
- **: \( p < 0.01 \)
- ***: \( p < 0.001 \)
- ****: \( p < 0.0001 \)

|          | Mean | P-value |
|----------|------|---------|
|          | WAP49 | WAP-F8 | WAP49 vs WAP-F8 |

#### A. Splenocytes

| Cytokine | Mean | P-value |
|----------|------|---------|
| IL-12    | 4.9  | 6.2     | NS         |
| IFN-\( \gamma \) | 32.3 | 29.1   | NS         |
| IL-4     | 19.7 | 33.1    | NS         |
| IL-5     | 312.8| 274.2   | NS         |
| IL-6     | 3.8  | 12.4    | NS         |
| IL-9     | 59.2 | 13.0    | ****       |
| IL-13    | 252.8| 472.7   | **         |

#### B. Mesenteric Lymph Nodes

| Cytokine | Mean | P-value |
|----------|------|---------|
| IL-12    | 14.2 | 5.5     | NS         |
| IFN-\( \gamma \) | 59.8 | 11.5   | NS         |
| IL-4     | 42.4 | 22.4    | NS         |
| IL-5     | 296.6| 162.9   | NS         |
| IL-6     | 1.8  | 0.8     | NS         |
| IL-9     | 101.4| 38.4    | NS         |
| IL-13    | 359.0| 178.3   | NS         |

#### C. Inguinal Lymph Nodes

| Cytokine | Mean | P-value |
|----------|------|---------|
| IL-12    | 21.175| 0       | NS         |
| IFN-\( \gamma \) | 80.4 | 0     | NS         |
| IL-4     | 3.0  | 4.1     | NS         |
| IL-5     | 115.2| 112.4   | NS         |
| IL-6     | 20.1 | 1.3     | NS         |
| IL-9     | 13.0 | 7.2     | NS         |
| IL-13    | 161.4| 66.9    | NS         |
Sera from mice immunized with r*Tm*-WAP49 or r*Tm*-WAP-F8+Na-GST-1 recognize native Tm-WAP protein

Serum samples from mice immunized with the various *T. muris*-derived immunogens (r*Tm*-WAP49, r*Tm*-WAP-F8+Na-GST-1, and r*Tm*-CAP-1) were tested for their antibody recognition of Tm-ES by ELISA and endpoint titters were determined (Figure 15A). We observed that both r*Tm*-WAP49 and r*Tm*-WAP-F8+Na-GST-1 exhibited significantly greater (~100-fold) recognition of Tm-ES than r*Tm*-CAP-1. Furthermore, antibody recognition of Tm-ES by r*Tm*-WAP49 and r*Tm*-WAP-F8+Na-GST-1 were similar to that of Tm-ES antisera, suggesting Tm-WAP as an immunodominant antigen in the ES products of the *T. muris* adult worm. We also performed western blot analyses of multiple *T. muris* products using serum samples from mice immunized with r*Tm*-WAP49, r*Tm*-WAP-F8+Na-GST-1, or Tm-ES (Figure 15B). We observed that mouse anti-Tm-ES serum originally used for the immunoscreening of the *T. muris* cDNA library clearly identified 49kd and 31kd protein bands corresponding to r*Tm*-WAP49 and r*Tm*-WAP-F8+Na-GST-1, respectively. The mouse anti-r*Tm*-WAP49 sera recognized r*Tm*-WAP49 and the WAP fragment within r*Tm*-WAP-F8+Na-GST-1, but not the rNa-GST-1 tag. Sera from mice immunized with r*Tm*-WAP-F8+Na-GST-1 recognized r*Tm*-WAP49 and rNa-GST-1. Importantly, sera from mice immunized with either r*Tm*-WAP49 or r*Tm*-WAP-F8+Na-GST-1 exhibited a similar recognition pattern of Tm-ES products, with several bands from 28-98 kDa range, corroborating findings that highly-conserved WAPs may be expressed as several secretory proteins of different molecular weights by both *T. muris* (127) and *T. trichiura* (21).
Figure 15. Cross-recognition of native and recombinant Tm-WAP and Tm-CAP-1 proteins. (A) Serum samples from mice immunized with r Tm-WAP49, r Tm-WAP-F8+Na-GST-1, and r Tm-CAP-1, and Tm-ES were tested for their IgG antibody recognition of Tm-ES by ELISA endpoint titers. Statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001. (B) Western blot analyses for the serological recognition and cross-recognition of r Tm-WAP49, r Tm-WAP-F8+Na-GST-1, and Tm-ES. In each panel, the lane numbers on the top represent: (1) SeeBlue pre-stained protein marker; (2) T. muris adult homogenate; (3) T. muris ES; (4) Recombinant Tm-WAP49 expressed in P. pastoris; (5) Recombinant Tm-WAP-F8+Na-GST-1 fusion protein expressed in E. coli; (6) Recombinant Na-GST-1 expressed in P. pastoris. The SDS-PAGE was performed using 4-20% gradient NuPAGE/MES gel. The blots were incubated with different mouse anti-sera (1:3,000) as shown under each panel. For WB with anti-Tm-ES, the loaded amount of protein was adjusted for lane 2 to 6 as 2.0 µg, 500 ng, 1.0 µg, 400 ng and 400 ng. The nitrocellulose membranes of anti-r Tm-WAP-F8+Na-GST-1 and anti-Tm-ES were cut just prior to ECL substrate reaction between lanes 4-5 and 3-4, respectively, to perform different lengths of exposure time to allow for the detection of the signal.
Recombinant *Tm*-WAP protein does not elicit antigen-specific IgE response

The generation of antigen-specific IgE antibodies secondary to vaccination or pre-existing in the population would likely impair the protective efficacy of immunization with that specific candidate vaccine (137). In response to a systemic vaccination these circulating parasite-specific IgE antibodies, if induced, can lead to a deleterious allergic response, as seen with the hookworm *Na*-ASP-2 vaccine (138). Serum-specific IgE to total IgE ratio is a clinical indicator of allergen-specific response (139, 140). We observed that vaccination with either *r Tm*-WAP49 or *r Tm*-WAP-F8+*Na*-GST-1 did not lead to any significant serum-specific IgE to total IgE ratio compared to Montanide ISA 720 (Figure 16). Ratios fell below a cutoff defined by the average values observed analyzing serum samples from control group of mice immunized with PBS. Additionally, *Tm*-ES, which contains the immunodominant native *Tm*-WAP, did not induce an IgE response.

![Figure 16. Analyses of serum IgE levels in mice immunized with r Tm-WAP49 and r Tm-WAP-F8+Na-GST-1.](image)

Serum levels of antigen-specific IgE were measured by ELISA and the total IgE concentrations were determined using a standard curve and shown as an antigen-specific IgE to total IgE ratio. Mice vaccinated with Montanide ISA 720 were used as negative controls. Cutoff (black dashed line) was defined by the average ratio induced by PBS.
3.4 Discussion:

In this study we demonstrated that Tm-WAP is a TH2 immunogen that is protective against T. muris challenge. Immunization with either the near full-length Tm-WAP (rTm-WAP49) or Tm-WAP fragment fusion protein (rTm-WAP-F8+Na-GST-1) led to a significant reduction in worm burden after T. muris challenge. Interestingly, relative protection after immunization with rTm-WAP49, was greater but not significantly different from that observed after immunization with rTm-WAP-F8+Na-GST-1.

Antibody recognition of both rTm-WAP49 protein and the fragment rTm-WAP-F8 by Tm-ES antisera and the reciprocal recognition of native Tm-WAP in Tm-ES by anti-rTm-WAP49 and anti-rTm-WAP-F8+Na-GST-1 sera, suggest that both candidates, as well as Tm-WAP in the Tm-ES, contain epitopes with conserved antigenicity.

Critical cellular mediators of protection against T. muris identified in our studies are the TH2 cytokines IL-4, IL-9, and IL-13. In this regard, it is well-established that the TH2 cytokine IL-4 is a critical mediator to enhance antibody responses (87), while IL-13 is recognized for its role in promoting worm expulsion through goblet cell expansion, mucin production, and smooth muscle hypercontractility (87, 89). Similarly, IL-9 mediates smooth muscle hypercontractility, as well as tissue mast cell maturation (88, 89). We identify a strong TH2 response at multiple key secondary lymphoid sites, including the parasite-draining MLNs, vaccine-draining ILNs, and the spleen. Given the important roles for these TH2 cytokines in the infection process of the worm, our results demonstrating the protective immunity elicited by the near full-length recombinant WAP protein, or the highly conserved fragment 8 of the WAP, strongly support their potential as vaccine candidates.

In contrast to the protective efficacy of the WAP, Tm-CAP-1, the second most highly expressed protein identified in our studies from the excreted-secreted products of T. muris, was ineffective in inducing strong type 2 immune response as well as protection. In addition, while
antisera from \textit{rTm-CAP-1} vaccinated mice did recognize \textit{Tm-ES} antigen by ELISA, it had nearly a 100-fold less recognition relative to either recombinant \textit{Tm-WAP} protein, which was identical to \textit{Tm-ES} antisera recognition of itself. This is surprising, given that literature reports identify the CAP-domain family (also known as SCP/TAPS) as a promising vaccine target due to its abundance in the secretome of whipworm and other soil-transmitted helminths (126, 127). Additional studies testing different immunization regimens and/or adjuvant formulations should inform whether or not the CAP-1 protein can induce TH2 immunity and protective efficacy. However, since \textit{rTm-CAP-1} protein was not found to be protective in our murine model, it was not included for the detailed biophysical characterization (Chapter 4) or evaluation of human immune recognition of protective \textit{T. muris} protein(s).

Pre-formed IgE antibodies to recombinant \textit{Na-ASP-2} in vaccine recipients from hookworm endemic regions are thought to have induced generalized urticaria, halting the vaccine’s development (141). In this regard, after three immunizations with either of the two recombinant WAP proteins we did not observe the parasite-specific IgE to total IgE ratio to be above background levels. Future studies evaluating the potential of the recombinant WAP proteins as vaccine candidates should determine whether natural infection with \textit{T. trichiura} generates pre-formed IgE antibodies that recognize the recombinant \textit{Tm-WAP} proteins.

In summary, we have developed two recombinant derivatives of \textit{Tm-WAP}, \textit{rTm-WAP-F8+Na-GST-1} and \textit{rTm-WAP49}, which are both protective in the AKR animal model. Furthermore, we demonstrate that both proteins generate a strong type 2 immune response, similar to the highly protective \textit{Tm-ES}. Future evaluations will require verification that \textit{Na-GST-1} fused with \textit{Tm-WAP-F8} retains protective efficacy as an individual candidate against hookworm, supporting the potential to developing pan-anthelminthic vaccine (108, 142, 143).
Chapter IV

Characterization and localization of protective *T. muris* proteins.
4.1 Introduction

As described in chapter II of this dissertation, we identified candidate antigens by screening the *T. muris* cDNA library with protective sera generated against *Tm*-ES. Using a whipworm animal model, we immunized mice with candidate antigens to evaluate immunity, as well as protection against subsequent *T. muris* challenge infection (Chapter III). Data from these studies identified *Tm*-WAP, derived from the *Tm*-ES, as a strong TH2 immunogen that elicits protection when administered as a recombinant near full-length (*rTm*-WAP49) or fragment-fusion protein (*rTm*-WAP-F8+Na-GST-1) co-formulated with Montanide ISA 720 adjuvant. Based on these promising results, we designed a series of analytical studies to determine the stability, homogeneity, and purity of *rTm*-WAP49 and *rTm*-WAP-F8+Na-GST-1 (Figure 17). Here we show that both these recombinant forms of *Tm*-WAP exhibit high degree of thermostability and are homogeneous with one major peak by ultra performance liquid chromatography (UPLC).

Sequence analysis of *Tm*-WAP revealed that it was likely a secretory protein (Chapter II). Given that *Tm*-WAP was identified through screening with *Tm*-ES, we hypothesized that *Tm*-WAP was secreted from the stichosome of *T. muris*. To test this hypothesis, we employed antiserum from mice immunized with *rTm*-WAP49 for immunofluorescence analyses of cecum tissue sections from mice infected with *T. muris*. We observed that antiserum from mice immunized with *rTm*-WAP49 localized the native WAP to the stichosome of *T. muris* within the infected mouse cecum tissues.
4.2 Materials and Methods

Immunofluorescence staining of mouse cecum

Immunofluorescent staining for Tm-WAP in adult *Trichuris muris* was performed using the M.O.M.™ Immunodetection Kit (Vector Labs, USA). In brief, SCID mice were sacrificed on day 42 post *T. muris* infection. Intestinal tissue with worms embedded were fixed in paraffin and sectioned. Primary staining was performed with PBS, naïve sera (1:100), or anti-\(rTm\)-WAP49 (1:100). Secondary staining was with FITC-conjugated goat anti-mouse IgG (1:500). For staining the cellular nuclei DAPI was embedded in the mounting medium.

Circular Dichroism

Circular dichroism (CD) spectra were used to predict the secondary structure of \(rTm\)-WAP49, \(rTm\)-WAP-F8+Na-GST-1 and \(rNa\)-GST-1. Samples were prepared at a protein concentration of 0.02 mg/mL in 10 mM phosphate buffer. The CD spectra were recorded with a Jasco J-1500 s spectrophotometer (Jasco Inc., USA), scanning from 190 nm to 300 nm at 50 nm/min with a bandwidth of 1 nm and response time of 1 second. Experiments were performed using a quartz cuvette with a path length of 1 cm at 25 °C. Six scans were averaged and compared to a baseline of buffer-only sample. CDPro software (Colorado State Univ., USA) was used to predict secondary structure against reference sets SP43, SDP48 and SMP56 and analyzed by CONTIN and CDSSTR, as previously described (144). In addition, CD spectra were collected as a function of temperature to determine the thermodynamics of protein unfolding. The samples were heated form 25°C to 90°C increasing the temperature stepwise at 1°C/data point. Each data point was determined as the average of two scans.

Ultra performance liquid chromatography

Ultra performance liquid chromatography (UPLC) was used to determine the size of the proteins in solution. The system consisted of a Waters ACQUITY UPLC® system with PDA detector (Waters, USA) fitted with an ACQUITY UPLC Protein BEH SEC 200Å column, designed
to characterize proteins ranging in molecular weight from 10 kDa–450 kDa. We used 2 µg each of \( rTm\)-WAP49, \( rTm\)-WAP-F8+Na-GST-1 and \( rNa\)-GST-1 to inject into a TSK gel Super SW2000 column (TOSOH Biosciences, USA) and eluted at 0.3 ml/min isocratically with PBS (pH 7.4) for 9 min. Absorbance was continuously measured at 220 nm. A gel filtration protein standard (BioRad, USA) was used to generate a calibration curve. The standard includes 5 globular proteins: Thyroglobulin (bovine), Gamma globulin (bovine), Ovalbumin (chicken), Myoglobin (horse), and Vitamin B12, with respective MW of 670 kDa, 158 kDa, 44 kDa, 17kDa and 1.35 kDa.

**High performance liquid chromatography with fractional collection**

High performance liquid chromatography (HPLC) was used to collect fractions of the different sizes of \( rTm\)-WAP49. The system consisted of an Agilent 1260 Infinity series HPLC, coupled with a UV detector (Agilent, USA), and a 1260 Infinity II Analytical-Scale Fraction Collector (Agilent). An aliquot of 200 µg of \( rTm\)-WAP49 was injected into a TSK gel Super SW2000 column (TOSOH Biosciences, USA) and eluted at 0.5 ml/min isocratically with PBS pH 7.4 for 35 min. After 10 min all fractions of 250 µL were collected in a 96-well plate.

**Antibody analysis**

Serum samples from mice immunized with \( Tm\)-ES and \( rTm\)-WAP49 were prepared from blood by centrifugation in serum-separating tube (Sarstedt, Germany) at 10,000 g for 5 minutes and stored at -80°C until assay. Samples were evaluated for antigen-specific immunoglobulins, IgG1 and IgG2a (Lifespan Biosciences, USA) by a modified indirect enzyme-linked immunosorbent assay (ELISA), as described previously (131). Concentration of \( rTm\)-WAP49 (0.375 µg/mL) used for ELISA was selected based on pretested optimal signal/noise ratio. The fractionally-collected \( rTm\)-WAP49 was quantified by NanoDrop A205 Protein Quantification (Thermo Fisher Scientific, USA) and used at 0.375 µg/mL to coat the ELISA plates. Antibody
absorbance was measured by dual wavelength analysis at test wavelength of 450 nm and the reference wavelength of 630 nm using a spectrophotometer (BioTek, USA).
4.3 Results

Native Tm-WAP protein localizes to the stichosome organ of T. muris

Over the past 20 years, the Trichuris spp. stichosome has been of particular interest due to its pathogenic role in facilitating intercellular existence within the host intestinal epithelial layer (1, 14, 21, 145). To determine whether Tm-WAP is stichosome-derived, we used anti-rTm-WAP49 mouse sera to perform fluorescent immunohistochemistry (IHC) of adult T. muris embedded in the caecal tissue of SCID mice on day 42 post-infection with 300 embryonated eggs (visualized by DAPI nuclear stain). Tissue cross-sections revealed an anti-Tm-WAP staining in the tissue-embedded stichosome of T. muris (Figure 17) with no discernable staining in the posterior, non-stichosome end of the worm. A predominance of the staining occurred in a ring-like, granular pattern (red arrow, Figure 17C) beneath the cuticle of the helminth. Directly adjacent to the outer layer of the helminth cuticle, we observed strong anti-Tm-WAP binding (white arrow, Figure 17C), suggesting secretion of the WAP protein into the mouse caecal intraluminal space. Of note, in certain cross sections, there were delineated structures with granular anti-Tm-WAP staining (yellow arrow, Figure 17B) within the stichosome. A similar pattern of variable distribution of antigen-specific secretory granules was previously described in the stichosome of T. spiralis (146).
Far-UV circular dichroism (CD) was used to determine the secondary structural components of rTm-WAP49 and rTm-WAP-F8+Na-GST-1. The rNa-GST-1 tag protein, which comprises 80% of the WAP fragment fusion protein (rTm-WAP-F8+Na-GST-1) and has been extensively biophysically characterized as hookworm vaccine candidate, was evaluated as a control (108, 142). Different secondary structural features, such as α-helices, beta-pleated sheets, and turns/loops, can be interpreted from characteristic positive and negative bands along the CD spectra (147–149). Additionally, given that protein structure is dependent on its environment, temperature, stability, and excipient (small molecule) interactions can be evaluated using CD (150).

The CD analyses revealed that rTm-WAP49 consists of 65% turns and loops, 30% beta-pleated sheets, and 5% α-helices (Figure 18). These values differ significantly from the predicted secondary structure by Phyre2, a protein modeling and prediction software (151), which projected 94% turns and loops, 0% beta-pleated sheets and 5% α-helices. The rTm-WAP-F8+Na-GST-1 protein, on the other hand, is comprised of 28% turns and loops, 13% beta-pleated sheets, and 59% α-helices by CD, which overlaps considerably with the predicted Phyre2 structure, at 34%, 7%, and 59%, respectively. The rNa-GST-1 tag protein had similar secondary structure as rTm-WAP-F8+Na-GST-1, with 16% turns and loops, 12% beta-pleated sheets, and 72% α-helices by CD, and a similar 28%, 10%, 62%, respectively, Phyre2 structural prediction.
To evaluate the denaturation characteristics of \( rTm-WAP49 \) and \( rTm-WAP-F8+Na-GST-1 \) proteins by CD, the molar ellipticity at 208 nm was measured over an increasing temperature range from 25 to 83°C, as previously described (152). The data was normalized between 1 (folded) and 0 (unfolded) is presented in Figure 19. Data was normalized as follows:

**Figure 18.** Secondary structural components of \( rTm-WAP49 \), \( rTm-WAP-F8+Na-GST-1 \), and \( rNa-GST-1 \). Circular dichroism (CD) spectra were scanned from 190 nm to 300 nm at constant temperature of 25 °C. Secondary structure was predicted using the CDPro software by comparing with three reference sets (SP43, SDP48 and SMP56) and using two data fitting programs (CONTIN and CDSSTR) and grouped into the categories helices, beta sheets, and turns and loops. Experimental CD data was compared to Phyre 2 predicted structure based on amino acid sequence.
Relative CD = \( \frac{|Y[\theta]_{\text{exp}}| - |Y[\theta]_{\text{min}}|}{|Y[\theta]_{\text{max}}| - |Y[\theta]_{\text{min}}|} \)

Where \( Y[\theta]_{\text{exp}} \) is the experimental molar ellipticity (degrees x cm\(^2\)/dmol), and \( Y[\theta]_{\text{min}} \) and \( Y[\theta]_{\text{max}} \) represent the minimum and maximum molar ellipticity, respectively. As described earlier, \( r\text{Na-GST-1} \) serves as a control reagent. All proteins were evaluated in phosphate buffered saline (PBS) at a concentration of 0.02 mg/mL for both \( r\text{Na-GST-1} \) and \( r\text{Tm-WAP-F8+Na-GST-1} \) had a melting temperature of 55°C and 58°C, respectively. The \( r\text{Tm-WAP49} \) proteins, however, had remarkable thermostability with a melting temperature of 75°C. The sharp denaturation of \( r\text{Tm-WAP49} \) and \( r\text{Tm-WAP-F8+Na-GST-1} \) is indicative of a single, well-defined low energy state that keeps the protein folded.

**Figure 19. Thermodynamics of protein unfolding of \( r\text{Tm-WAP49} \), \( r\text{Tm-WAP-F8+Na-GST-1} \), and \( r\text{Na-GST-1} \).** The samples were heated from 25°C to 90°C increasing the temperature stepwise at 1°C/data point and measured at 208 nm. Each data point was determined as the average after two scans.
Analysis of purity, sizing, and homogeneity of recombinant Tm-WAPs

To evaluate the biophysical integrity of \( rTm\)-WAP49 and \( rTm\)-WAP-F8+Na-GST-1 in solution, we used gel filtration liquid chromatography (LC) to study the size distribution of the proteins (153, 154). We initially characterized the recombinant Tm-WAPs by ultra performance liquid chromatography (UPLC) using an ACQUITY UPLC Protein BEH SEC 200Å column, which is optimized to separate proteins between 10 kDa–450 kDa. A molecular weight standard was run over the column, to create a calibration curve and \( rNa\)-GST-1 served as a continued protein of comparison to \( rTm\)-WAP-F8+Na-GST-1. By UPLC, \( rNa\)-GST-1 was a dimer protein, with a sharp single peak at molecular weight 43 kDa (\( rNa\)-GST-1 “a”, Figure 20) confirming previous reports (155). As can be observed, the main peak of the chromatogram of \( rTm\)-WAP-F8+Na-GST-1 (\( rTm\)-WAP-F8+Na-GST-1 “b”, Figure 20) is eluting only slightly earlier and has an estimated molecular weight of 51 kDa. As can be observed, the peak width of \( rTm\)-WAP-F8+Na-GST-1 is wider than the peak of \( rNa\)-GST-1, which is not unusual for a fusion protein. \( rTm\)-WAP49, on the other hand, showed multiple large peaks at (“a”) 210 kDa, (“b”) 65 kDa, and (“c”) 3.4 kDa (\( rTm\)-WAP49, Figure 20), suggestive of either protein oligomerization or aggregation. However, it should be noted that, due to the lack of any aromatic amino acids (tryptophan or tyrosine) in the WAP fragment, all samples were measured using UV absorption at 220nm. At this wavelength any possible trace amounts of chromophores or organic compounds (e.g. amino acids) from the production process can be detected, likely causing peak “c” at 3.4 kDa. They are not likely due to protein degradation, as fractionally collected peak “c” did not show any protein bands by Coomassie staining on SDS PAGE.
To further evaluate the multiple peaks identified in the rTm-WAP49, the protein was denatured by sodium dodecyl sulfate and separated by molecular weight using polyacrylamide gel electrophoresis (SDS-PAGE) and stained by Coomassie Brilliant Blue (Figure 21), as previously described (156). rTm-WAP49 reduced with 2-Mercaptoethanol (lanes 3-4), reveal a
single 49 kDa smeared protein. The non-reduced rTm-WAP49 reveal 3 distinct bands between 62 and 188 kDa.

**Figure 21. Multiple protein bands of non-reduced rTm-WAP49.** In each panel, the lane numbers on the top represent: (M) SeeBlue pre-stained protein marker; (1-2) BSA control at 1 µg and 2 µg; (3-4) reduced recombinant Tm-WAP49 at 1 µg and 2 µg; and (5-6) non-reduced recombinant Tm-WAP49 at 1 µg and 2 µg. The SDS-PAGE was performed using 4-20% NuPAGE/MES gel and stained with Coomassie Brilliant Blue.

To determine whether the multiple rTm-WAP49 peaks found by UPLC and visualized by SDS-PAGE were due to protein instability, rTm-WAP49 was fractionally collected by size-exclusion high performance liquid chromatography (HPLC) between 1A through 8H. Size-exclusion HPLC is similar to the chromatogram of the size-exclusion UPLC assay, with a slightly lower resolution, but connected to a fraction collector. Based on a gel filtration MW standard curve, fractions 7B, 6A, and 5A relate to protein samples with average respective molecular weights of 49 kDa, 96 kDa, and 147 kDa (**Figure 22A**). After collection of multiple fractions 7B, 6A, and 5A, they were re-run through the HPLC, and were found to have solitary peaks,
suggesting that individual fractions were stable in various oligomer states, likely as a monomer, a dimer, and a trimer state, and did not further aggregate. Fractions 4A through 7D were evaluated for variations in immune recognition. Serum generated by mice immunized with rTm-WAP49 showed equal recognition of the various fractions and unfractionated rTm-WAP49 (Figure 22B). Serum generated by mice immunized with Tm-ES, however, had increasing recognition with as the fraction size increased (Figure 22C).
Figure 22. Fractional collection of different size rTm-WAP49 and their conserved recognition by rTm-WAP49 and Tm-ES antisera. (A) High performance liquid chromatography (HPLC) was used to collect varying sizes of rTm-WAP49 between fractions 4A and 7D (top). Fractions 7B, 6A, and 5A were reanalyzed by HPLC (middle) and sized by molecular weight using a Bio-Rad protein gel standard (bottom). (B) rTm-WAP antisera and (C) Tm-ES antisera were used to evaluate immune recognition of rTm-WAP49 fractions. Total IgG was measured by ELISA at plotted based on optical density (OD). Unfractionated rTm-WAP49 was used as a positive control antigen and baseline, and naïve sera from unvaccinated mice was used a negative control.
4.5 Discussion

Using rTm-WAP49 antisera, native Tm-WAP protein was localized to the stichosome organ of T. muris. Protection generated in mice immunized against Tm-WAP further supports previous studies suggesting that the stichosome plays a critical role in helminth pathogenicity (70, 71). The homologous stichosome-derived secretory Trichuris trichiura WAPs, Tt52 and Tt95, have been described for their ability to generate pores in a planar lipid bilayer (21–23). Future studies testing the ability of Tm-WAP antisera to inhibit pore-formation, preventing stichosome penetration and maintenance in the colonic mucosa would be important to further delineate the mechanism underlying the protective efficacy observed for the recombinant Tm-WAP.

To evaluate the protein characteristics and stability of rTm-WAP-F8+Na-GST-1 and rTm-WAP49, we conducted a biophysical assessment of both candidates using multiple analytical techniques such as SDS-PAGE, circular dichroism (CD), and liquid chromatography. Collectively, this data will provide specific information concerning the physical state of each protein, creating a baseline for further stability testing.

We observed that rTm-WAP-F8+Na-GST-1 shared biophysical characteristics of both rTm-WAP49 and rNa-GST-1. Since rTm-WAP-F8+Na-GST-1 is 80% composed of Na-GST-1, unsurprisingly, all biophysical characteristics we measured had greater similarity to rNa-GST-1. By CD, the secondary structure of rTm-WAP-F8+Na-GST-1 revealed it was primarily composed of α-helices, like rNa-GST-1. However, rTm-WAP-F8+Na-GST-1 did retain the characteristic of additional turns and loops, as seen with rTm-WAP49. When evaluating the thermodynamics of protein unfolding by CD, rTm-WAP-F8+Na-GST-1 exhibited a slightly increased melting temperature compared to rNa-GST-1. Characterization by UPLC further supported that rTm-WAP-F8+Na-GST-1 shared high similarity with rNa-GST-1. Overall, the highly conserved biophysical properties between rTm-WAP-F8+Na-GST-1 and rNa-GST-1, coupled with the conserved antigenicity by western blot, suggest that rTm-WAP-F8+Na-GST-1 may retain enough
protein similarity to vaccine candidate rNa-GST-1 (108), to have conserved cross-protection against hookworm challenge.

By CD, the secondary structure of rTm-WAP49 revealed it was primarily composed of turns and loops, followed by beta-sheets. Interestingly, rTm-WAP49 had remarkable thermostability, with a melting temperature of 75°C, and uniformity with a sharp denaturing curve. The high stability is probably associated with the disulfide core domain of the whey acidic protein family. Given that Tm-WAP was identified from among the excreted-secreted products of T. muris, and our fluorescent immunohistochemistry analyses suggested secretion into the colonic mucosa, extreme protein stability may be necessary for preserved function in the harsh intestinal environment. By UPLC, at 220nm, rTm-WAP49 showed varying size between 65kDa and 210 kDa. Given the single band seen by SDS-PAGE when reduced and denatured, along with the sharp denaturing curve by CD, we suspect that rTm-WAP49 exists in multimers. Fractions collected by HPLC showed sharp protein peaks of each fragment when retested on HPLC. This indicates protein oligomerization, as opposed to uncontrolled protein aggregation due to instability. With 43 cysteine residues in the protein, one possibility is disulfide bond formation leading to a mix of monomeric, dimeric, and trimeric species. To determine whether or not this affected immunogenicity, we tested fractions for recognition by IgG from mice immunized with unfractionated rTm-WAP49 or Tm-ES. There was no difference in recognition by rTm-WAP49 antisera compared to naïve sera. While all rTm-WAP49 fractions were recognized by Tm-ES antisera, compared to naïve sera, there was an increased recognition of larger, multimeric fractions. Interestingly, in Chapter III we demonstrated that a western blot of Tm-ES with rTm-WAP49 antisera reveals multiple bands with cross recognition. This suggests that native T. muris WAP may exist as multimers or conserved gene duplications, similar to that reported for T. trichiura WAP (21). Nonetheless, rTm-WAP49 may need to be re-formulated or re-engineered to evaluate the role of multimers on immunogenicity and efficacy.
Chapter V

*In vitro* evaluation of human immune recognition for the *T. muris* recombinant proteins exhibiting protective efficacy
5.1 Introduction

The ultimate goal of the research presented in this dissertation was to identify candidate *T. muris* antigens protective in an animal model and to explore immune mechanisms of protection. Given the immunogenicity and protective efficacy of *Tm*-WAP demonstrated from data presented in Chapters II and III of this dissertation, we explored the translational potential by testing immune recognition of *Tm*-WAP by serum and T cells from patients previously infected with *T. trichiura*. To achieve this, we implemented a cross-sectional human study of 236 healthy volunteers between the ages of 13 and 45 years from the rural region of Honduras. This age range was selected based on epidemiological data suggesting increased protection from trichuriasis (157). While prevalence of disease remained constant after 1 year of age, the worm burden, and subsequent associated morbidity, dramatically decreases beginning in early adolescence (157). This finding suggests that although the risk of exposure does not decrease with age, beginning in adolescents, the infected host is better able to control infection. We hypothesize this is due, at least in part, to an immune mediated mechanism of protection after multiple early exposures to the parasite.

Honduras is a low middle-income nation in Central American nation that is endemic with high rates of soil-transmitted helminths (STHs). Mass drug administration (MDA) in Honduras is conducted almost exclusively through the primary school system, providing coverage to nearly 70% of children in school (158). However, given the combination of the particularly low efficacy of MDA drugs against trichuriasis (106), the inability of the Honduran government to co-implement aggressive sanitary infrastructure (158), and the nearly 20% of students who drop-out of primary school in Honduras by the age of 15 and do not receive MDA (159), *T. trichiura* is still highly endemic in Honduras. This is particularly true in the rural regions of Honduras, which led us to selecting the remote area in and around the province of Colomoncagua, within the Department of Intibucá.
The first goal of the study was to identify the active prevalence of trichuriasis in the region, which has not been reported in this area to our knowledge. The second objective was to use serum samples and peripheral blood mononuclear cells (PBMC) from participants to determine immune recognition of rTm-WAP49 from this endemic region.
5.2 Materials and Methods

Ethics statement

All human sample collection in Honduras was conducted in accordance with University of Texas Health Science Center at Houston Institutional Review Board (IRB) (approved protocol HSC-MS-14-0035), Baylor College of Medicine IRB (approved protocol H-33704) and the National Autonomous University of Honduras (UNAH) Committee of Ethics Investigation (approved protocol No. 01-2014). Non-endemic control serum samples from participants living in the United States of America (USA) were collected and used in accordance with Baylor College of Medicine IRB (approved protocol H-35471). All human research was conducted in compliance with applicable United States and Honduran federal statutes and regulations relating to human research.

Study Population

We originally enrolled 30 adolescents (ages 13-17) and 53 adults (ages 18-45) in the municipality of Colomoncagua. Of these, 3 adults (age 45+) were discovered later to be outside the inclusion criteria for age range, and were removed from the study after being notified, and reported to the IRB. Subsequently, we enrolled an additional 83 adolescents and 73 adults. Together, the 113 adolescents and 123 adults constitute the 236 total participants evaluated in the study.

Inclusion Criteria:

Any adolescent (13-17) or adult (18-45) living within the municipality of Colomoncagua, deemed medically fit to donate stool and blood samples, was included after informed consent/assent was obtained.

Exclusion Criteria:
Any person given deworming medication (albendazole, mebendazole, levamisole, and pyrantel pamoate) in the past 6 months was excluded. Non-healthy participants, with the exception of symptoms related to a gastrointestinal parasitic infection, determined by one of our physicians, was excluded and instead offered medical evaluation separate to this research study by clinic staff. Pregnant women were excluded from the study. Given that a 10mL blood draw and providing a stool sample has little medical risk to the mother or fetus, as well as no effect on the study, questioning the female participant about their pregnancy status was ethically and medically sufficient as a means of exclusion.

Community leaders within the town of Santa Ana, where the Houston Shoulder to Shoulder Clinic is located, and surrounding communities within Colomancaaguia were contacted at the outset for their interest in participating in the study. Adults aged 18-45 years were asked to sign a consent form and adolescents aged 13 to 17 years were asked to provide assent with their parent or guardian singing a consent form, all in Spanish.

**Sample collection and processing**

Approximately 50g of fresh stool from each subject was used for DNA extraction by a modified MP FastDNA for Soil Kit (MP Biochemicals, USA), as previously described (160). 50 ul of extracted DNA samples were stored at -20°C until experimental analysis. 2 mL of blood were obtained by venipuncture into sodium heparin collection tubes (BD, USA) and immediately centrifuged at 10,000 g for 5 minutes to collect approximately 1 mL of serum and stored at -80°C until analysis. Due to the logistic difficulties associated with the limited resource setting, only 68 participants in 2016 were selected for peripheral blood mononuclear cells (PBMC) isolation. PBMCs were freshly isolated by Ficoll-Paque separation technique from approximately 8 mL of venous blood in sodium heparin collection tubes (BD, USA), as previously described (161), and stored as aliquots of 5-7 million cells per mL in freezing media (90% heat inactivated FBS [VWR, Radnor, PA] + 10% DMSO [Corning, USA]) at -80°C until experimental analysis.
Non-endemic serum samples from participants living in the USA were used as a negative control for antibody analysis. A total of 95 age-matched participants with no travel history to Trichuris endemic regions were selected among the -80°C banked samples.

**Real-time Polymerase Chain Reaction Assay**

Species-specific primers and TaqMan MGB probes (Thermo Fisher Scientific, USA) were used for the PCR analyses of DNA from the stool samples to determine the prevalence of helminths species: *Trichuris trichiura, Ascaris lumbricoides, Ancylostoma duodenale, Necator americanus, Strongyloides stercoralis*, and intestinal protozoal species *Cryptosporidium parvum/hominis, Giardia lamblia, and Entamoeba histolytica*. Primer sequences and detailed methodology were previously described (160).

**Antibody analysis**

Serum samples were evaluated in triplicate for antigen-specific immunoglobulins IgG (Lifespan Biosciences, USA) and IgE (Thermo Fisher Scientific, USA) by a modified indirect enzyme-linked immunosorbent assay (ELISA), as described previously (131). The individual wells of the ELISA plate were coated using a concentration of rTm-WAP49 at 0.375 µg/mL that was selected by pretesting for optimal signal/noise ratio. The absorbance was measured by dual wavelength analysis at test wavelength of 450 nm and the reference wavelength of 630 nm using a spectrophotometer (BioTek, USA).

**Cytokine analysis**

For the analyses of antigen-specific cytokine production, the frozen samples of PBMCs were first thawed in a 37°C water bath and washed in complete RMPI medium (RPMI 1640 with L-glutamine [VWR, USA], 10% heat inactivated FBS [VWR, Radnor, PA], and 1x pen/strep solution [Thermo Fisher Scientific, USA]) to remove residual DMSO. Subsequently, the PBMCs were resuspended in complete RMPI medium and incubated for 2 hours at 37°C before
determining the number of live cells using a Cellometer Auto 2000 Cell Viability Counter (Nexelcom, USA).

Cells were seeded in a 96-well U-bottom culture plate (Corning, USA) at 1x10^6 cells per well in 250 μl medium and restimulated with either rTm-WAP49 or Tm-ES at varying concentration at 37°C, 5% CO2 for varying time points. An unstimulated, media only negative control and a positive control stimulation with a mixture of 20 ng/mL PMA and 1 μg/mL Ionomycin were performed concurrently. Stimulated cells were pelleted by centrifugation at 300 x g for 5 min and the supernatants were collected for measuring cytokine production by Luminex analysis, as previously described (133), and cells were stained with fluorochrome-conjugated antibodies (Table 6) for determining cellular phenotype and function by flow cytometry analysis on the Attune NxT Flow Cytometer, as previously described (162).

| Target     | Fluorochrome          | Vendor (catalog #) |
|------------|-----------------------|--------------------|
| Viability  | Live/Dead Near IR     | Thermo Fisher (L23105) |
| CD3e       | Pacific Blue          | Biolegend (300330) |
| CD4        | FITC                  | eBioscience (11-0048-42) |
| CCR7 (CD197) | PerCP-Cy5.5        | RnD (L23105) |
| CD45RA     | Alexa Fluor 700       | Biolegend (304120) |
| CD25       | PE/Dazzle 594         | RnD (FAB197T-025) |
| IL-4       | Brilliant Violet 605  | Biolegend (500828) |
| IL-5       | APC                   | Biolegend (504306) |
| IL-13      | PE-Cy7                | Biolegend (501914) |
| IFN-γ      | Brilliant Violet 711  | BD (564793) |
| FoxP3      | PE                    | eBioscience (12-4776-42) |
| Ki-67      | Brilliant Violet 510  | Biolegend (50518) |

Table 6. List of fluorochrome-conjugated antibodies used for flow cytometry of human samples.
5.3 Results

Across two collection time points, we enrolled 236 participants between 13 and 45 years of age within the province of Colomoncagua, Honduras. Participants across 7 towns were characterized based on demographics, hematocrit (%), and relevant gastrointestinal (G.I.) symptoms (Table 7). The average age of participation was 23.2 with nearly 60% females. Overall, participants had normal hematocrits, with the average at 42.3%. However, over 40% of otherwise healthy volunteers had complaints of G.I. pain, with a subsequent 14% reporting diarrhea and 9% reporting constipation. Overall, there was not considerable difference in population characteristics or demographics across the towns in the province of Colomoncagua.

| Characteristic | Callejones (n = 6) | Colomoncagua (n = 92) | El Carrizal (n = 17) | El Hondable (n = 8) | Llano Grande (n = 11) | San Antonio Vados (n = 20) | Santa Ana (n = 82) | Total (n = 236) |
|----------------|--------------------|-----------------------|---------------------|---------------------|-----------------------|--------------------------|---------------------|-----------------|
| Sex            |                    |                       |                     |                     |                       |                          |                     |                 |
| Male           | 1                  | 39                    | 6                   | 5                   | 5                     | 7                        | 26                  | 89              |
| Female         | 5                  | 53                    | 11                  | 3                   | 6                     | 13                       | 56                  | 147             |
| Age (years)    | 14.0               | 19.3                  | 25.6                | 21.6                | 22.1                  | 22.1                     | 25.9                | 23.2            |
| Hematocrit (%) | 43.8               | 43.8                  | 41.8                | 41.4                | 43.1                  | 43.1                     | 40.6                | 42.3            |
| G.I. symptoms  |                    |                       |                     |                     |                       |                          |                     |                 |
| G.I. pain (%)  | 50%                | 38%                   | 30%                 | 60%                 | 43%                   | 60%                      | 28%                 | 44%             |
| Diarrhea (%)   | 33%                | 10%                   | 20%                 | 20%                 | 0%                    | 15%                      | 3%                  | 14%             |
| Constipation (%)| 17%                | 7%                    | 10%                 | 20%                 | 0%                    | 10%                      | 3%                  | 9%              |

Table 7. Demographic data and gastrointestinal symptoms of 236 participant in the province of Colomoncagua, Honduras. Demographic and basic questionnaire data was collected on healthy participants ages 13-45 years old across 7 towns within Colomoncagua. Sex, age, and hematocrit were measured for all 236 participants. Gastrointestinal (G.I.) symptoms questionnaire was only performed among the 156 participants recruited in 2016.
Prevalence of trichuriasis and other gastrointestinal parasites in study population

Fresh stool samples from participants were stored at 4°C and processed within 24hrs of collection. Extracted DNA from approximately 50g of stool was examined for parasite-specific DNA by qPCR. We analyzed for the 8 of the most common human G.I. parasites worldwide (160), including 5 helminths (trichuriasis, ascariasis, hookworm (both N. americanus and A. duodenale), as well as 3 protozoans (cryptosporidiosis, giardiasis, and amebiasis). Nearly 50% of the study population was infected with one or more screened G.I. parasites (Figure 23). To our surprise, only about 2% of participants were actively infected with T. trichiura. Reported prevalence in children in the surrounding areas is as high as 20% (158). Ascariasis and hookworm, on the other hand, had approximately 14% and 10% prevalence among participants.

Figure 23. Prevalence of 8 of the most common gastrointestinal parasites, including Trichuris trichiura, in the province of Colomoncagua, Honduras. Parasite DNA extracted from the stool of participants was measured by qPCR.
Serological recognition of r Tm-WAP49

After confirming active transmission of *T. trichiura* within Colomoncagua, we set out to measure serological recognition of r Tm-WAP49. To date, there are no commercially available specific serological tests for *T. trichiura*. To validate true reactivity, we used non-endemic serum from 95 age-matched individuals from USA with no travel history to whipworm endemic regions. Serum-specific IgG of r Tm-WAP49 (0.375 ug/mL) was measured for each sample, in triplicate, and plotted based on the degree of recognition on a histogram (Figure 2A). We identified a statistically significant difference (p<0.0001) in mean IgG recognition between the two populations as measured by ELISA. (Figure 2B).

To evaluate humoral allergenicity, we screened for serum-specific IgE recognition of r Tm-WAP49 (0.375 ug/mL) between the two populations and plotted along a histogram (Figure 25A). There was no statistically significant difference (p=0.1717) between the Honduras population and the non-endemic USA population (Figure 25B).
Figure 24. Total IgG sera recognition of rTm-WAP49. (A) Participants from the Trichuris trichiura endemic region of Colomoncagua, Honduras (n = 236) and compared to non-endemic, non-travelled, age-matched controls from the USA (n = 95). Results are plotted on a histogram based on the degree of total IgG recognition of rTm-WAP49 (0.375 µg/mL) by ELISA. (B) Unpaired t test with Welch’s correction comparing the IgG means of the two populations. Statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.
Unpaired t test with Welch’s correction

| Parameter                        | Value            |
|----------------------------------|------------------|
| P value                          | 0.1717           |
| P value summary                  | ns               |
| Significantly different (P < 0.05)? | No              |
| One- or two-tailed P value?      | Two-tailed       |

Welch-corrected t, df  
\[t=1.371 \text{ df}=253.8\]

How big is the difference?

| Description                               | Value          |
|-------------------------------------------|----------------|
| Mean ± SEM of USA (Control) Population    | 0.1053 ± 0.003619, n=95 |
| Mean ± SEM of Honduran Population         | 0.1301 ± 0.01775, n=236 |
| Difference between means                  | 0.02483 ± 0.01812 |
| 95% confidence interval                   | -0.01085 to 0.06051 |

Figure 25. Total IgE sera recognition of rTm-WAP49. Participants from the *Trichuris trichiura* endemic region of Colomoncagua, Honduras (n = 236) and compared to non-endemic, non-travelled, age-matched controls from the USA (n = 95). Results are plotted on a histogram based on the degree of IgE recognition of rTm-WAP49 (0.375 ug/mL) by ELISA. (B) Unpaired t test with Welch’s correction comparing the IgE means of the two populations. Statistical significance: *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001.
Recognition of rTm-WAP49 by T cells in the blood from *Trichuris trichiura* endemic patients

To determine whether T cells from Honduran participants recognized rTm-WAP49, we measured reactivity of peripheral blood mononuclear cells (PBMCs) to stimulation by flow cytometry and Luminex analyses. For flow cytometry, we developed a T cell gating strategy that evaluated for the expression of activation and proliferation markers along with production of T helper type 1 (TH1) and T helper type 2 (TH2) cytokines (Figure 26). Because of the difficulty in collecting PBMC samples in a resource-limited rural setting, we were only able to collect samples from 68 of the 236 participants. Assay optimization was focused on a further subset of participants with the highest serum IgG specific to rTm-WAP49.

Two participants (Participant 60 and 90) were initially selected based on their high IgG recognition of rTm-WAP49 and determined that their frozen PBMC to have lymphocyte viabilities greater than 85% (Table 8A). Aliquots of 250,000 live cells from each participant were stimulated with rTm-WAP49 at 50, 20, or 5 μg/mL for 24, 48, 72 or 96 hours (Table 8B). Additionally, Tm-ES was used as a stimulant for cells from Participant 90 following the same concentrations and incubation times as used for stimulation with rTm-WAP49. Antigen-specific stimulation was evaluated in terms of CD4+ T cell proliferation (Ki67+) or cytokine production (IL4+, IL-5+, IL-13+, and IFN-γ). Additionally, we evaluated CD4+CD25+FoxP3+ T regulatory (T reg) cells for activation (CD45RA+). We used PMA/I as a positive control reagent for stimulation and observed increased production of IL-4, IL-5, IL-13 and IFN-γ by the CD4+ T cells, as well as CD45RA+ T regs. However, cells from neither of the two participants showed expression of activation markers in response to stimulation with either rTm-WAP49 or Tm-ES.

Since T cell activation requires both antigen-stimulation as well as co-stimulation, we repeated the experiment, with the addition of anti-CD28/49D. In addition to monitoring the intracellular cytokine expression, we also evaluated the cytokines produced in the supernatants of activated cells by Luminex. Frozen cells from five participants with high anti-rTm-WAP49 IgG
levels and cell viabilities above 90% were selected for these assays (Table 9A). We used rTm-WAP49 (50 ug/mL) and Tm-ES (50 ug/mL) to stimulate 250,000 live cells for 12 h, following the protocols from the vendor of the anti-CD28/49D reagents. Additionally, PHA was used as a positive control that showed high levels of IL-4, IFN-γ and Ki67 in CD4+ T cells, as well as CD45RA expression on T regs (Table 9B). In all these assays, neither rTm-WAP49 nor Tm-ES induced cytokine responses by the CD4+ T cells in any of the participants.

For four of the five participants we had enough cells to concurrently detect TH2 (IL-4, IL-5, IL-13) and TH1 (IFN-γ) cytokines in the supernatants after stimulation using the Luminex analyses (Table 10). For this, aliquots of 250,000 live cells were co-stimulated with anti-CD28/CD49D and either media alone, Tm-ES (50 ug/mL), or rTm-WAP49 (50 ug/mL), and supernatant samples were collected at 36, 60, 84, 132 hours. We pooled cells from all four subjects for stimulation with PHA as a positive control and observed production of significant levels of IL-4, IL-5, IL-13, and IFN-γ, relative to media control and also cells stimulated with either rTm-WAP49 or Tm-ES. The later data suggests that PBMC from the subjects from the endemic area were not responsive to stimulation with rTm-WAP49 or Tm-ES, which may be either due to low sensitivity of the assays used or true low frequency of antigen-specific cells as recalled with the mouse antigens.
**Figure 26. T cell gating strategy and representative flow plots.** CD4+ T cells were gated from live CD3+ lymphocytes. Stimulated CD4+ T cells were further characterized by activation (Ki67+), TH2 cytokine production (IL4+, IL5+, and IL-13+), and TH1 cytokine production (IFN-γ). The CD4+ T regulatory cells were identified by dual staining as CD25+FoxP3+ and measured for activation after stimulation as CD45RA+. 
Table 8. T helper and T regulatory cell responses to stimulation with rTm-WAP49 determined by flow cytometry. (A) Two participants with high IgG recognition of rTm-WAP49 were selected for PBMC stimulation and optimization and measured for viability. (B) 250,000 live cells were stimulated with either rTm-WAP49 or Tm-ES at varying concentrations (5-50 μg/mL) and time of stimulation (24-96 hours). PMA/I and media alone were used as positive and negative controls, respectively. CD4+ T cell response was measured by intracellular staining for IL-4, IL-5, IL-13, IFN-γ, and proliferation with Ki67 staining. The CD4+CD25+FoxP3+ T regulatory cells were analyzed for activation based on CD45 expression.
Table 9: T helper and T regulatory cell responses to stimulation with rTm-WAP49 and Tm-ES after including costimulatory anti-CD28/CD49D for flow cytometry. (A) Five participants with high IgG recognition of rTm-WAP49 were selected for PBMC stimulation and optimization and measured for viability. (B) 250,00 live cells were stimulated with either rTm-WAP49 or Tm-ES at 50 μg/mL and co-stimulatory molecules anti-CD28/CD49D for 12 hours. PHA and media with co-stimulatory molecules anti-CD28/CD49D were used as positive and negative controls, respectively. CD4+ T cell response was measured by intracellular staining for IL-4, IL-5, IL-13, IFN-γ, and proliferation with Ki67 staining. The CD4+CD25+FoxP3+ T regulatory cells were analyzed for activation based onCD45 expression. Values for all five participants were averaged and compared to those of control subjects.
Table 10: Cytokine response to stimulation with \( rTm\)-WAP49 and \( Tm\)-ES after including costimulatory anti-CD28/CD49D for Luminex analysis. Cells from four participants with the high IgG recognition of \( rTm\)-WAP49 were measured for cytokine production in supernatant after stimulation. 250.00 live cells were restimulated with either \( rTm\)-WAP49 or \( Tm\)-ES at 50 ug/mL and co-stimulatory molecules anti-CD28/CD49. Samples of supernatant were taken at 34, 60, 84, and 132 hours. Supernatants were measured for IL-4, IL-5, IL-13, and IFN-\( \gamma \) concentrations by Luminex analysis. Values for all four participants were averaged and compared to those of controls.

| ID            | Antigen | Dose (ug/mL) | IL-4 | IL-5 | IL-13 | IFN-\( \gamma \) |
|---------------|---------|--------------|------|------|-------|-----------------|
| High Control  | N/A     | N/A          | 2988 | 276  | 585.5 | 5098            |
| Low Control   | N/A     | N/A          | 82.77| 8.03 | 21.2  | 192.9           |
| **Stimulation 36 hours** | | | | | | |
| Pooled       | PHA     | 1x           | 3050.0| 11.3| 30.1   | 94.7            |
| Media        | N/A     | N/A          | 0.2  | 0.4  | 3.6    | 10.4            |
| WAP          | 50      | 0.2          | 5.8  | 5.8  | 5.8    | 3.5             |
| Participant 41| ES      | 50           | 0.2  | 0.3  | 4.4    | 10.4            |
| Participant 60| ES      | 50           | 11.9 | 2.2  | 5.8    | 10.4            |
| Participant 68| ES      | 50           | 0.2  | 0.2  | 5.2    | 3.5             |
| Participant 102| ES     | 50           | 2.1  | 0.8  | 11.2   | 14.4            |
| Participant 102| WAP    | 50           | 0.2  | 0.6  | 5.5    | 3.5             |
| **Averaged** | Media   | N/A          | 1.2  | 0.2  | 4.1    | 6.9             |
| Media        | N/A     | N/A          | 3.6  | 0.3  | 7.0    | 7.9             |
| WAP          | 50      | 0.3          | 6.3  | 0.4  | 5.4    | 9.7             |
| **Stimulation 60 hours** | | | | | | |
| Pooled       | PHA     | 1x           | 3000.0| 4.3  | 41.1   | 217.3           |
| Media        | N/A     | N/A          | 14.5 | 0.2  | 6.9    | 23.2            |
| WAP          | 50      | 10.9         | 30.7 | 30.7 | 88.8   | 66.6            |
| Participant 41| ES      | 50           | 36.7 | 0.1  | 6.3    | 16.7            |
| Participant 60| ES      | 50           | 48.3 | 0.1  | 83.3   | 53.0            |
| Participant 68| ES      | 50           | 144.2| 0.1  | 6.3    | 29.9            |
| Participant 102| ES     | 50           | 0.2  | 0.1  | 8.3    | 19.0            |
| Participant 102| WAP    | 50           | 58.1 | 0.1  | 15.6   | 49.1            |
| Participant 102| WAP    | 50           | 22.2 | 0.1  | 90.3   | 98.3            |
| Participant 102| WAP    | 50           | 43.4 | 0.1  | 118.3  | 144.9           |
| **Averaged** | Media   | N/A          | 6.1  | 0.2  | 22.6   | 56.0            |
| Media        | N/A     | N/A          | 20.4 | 0.1  | 38.3   | 63.5            |
| WAP          | 50      | 0.7          | 36.5 | 0.1  | 36.6   | 60.1            |
| **Stimulation 132 hours** | | | | | | |
| Pooled       | PHA     | 1x           | 3000.0| 4.3  | 41.1   | 217.3           |
| Media        | N/A     | N/A          | 14.5 | 0.2  | 6.9    | 23.2            |
| WAP          | 50      | 10.9         | 30.7 | 30.7 | 88.8   | 66.6            |
| Participant 41| ES      | 50           | 36.7 | 0.1  | 6.3    | 16.7            |
| Participant 60| ES      | 50           | 48.3 | 0.1  | 83.3   | 53.0            |
| Participant 68| ES      | 50           | 144.2| 0.1  | 6.3    | 29.9            |
| Participant 102| ES     | 50           | 0.2  | 0.1  | 8.3    | 19.0            |
| Participant 102| WAP    | 50           | 58.1 | 0.1  | 15.6   | 49.1            |
| Participant 102| WAP    | 50           | 22.2 | 0.1  | 90.3   | 98.3            |
| Participant 102| WAP    | 50           | 43.4 | 0.1  | 118.3  | 144.9           |
| **Averaged** | Media   | N/A          | 6.1  | 0.2  | 22.6   | 56.0            |
| Media        | N/A     | N/A          | 20.4 | 0.1  | 38.3   | 63.5            |
| WAP          | 50      | 0.7          | 36.5 | 0.1  | 36.6   | 60.1            |

### Table 10: Cytokine response to stimulation with \( rTm\)-WAP49 and \( Tm\)-ES after including costimulatory anti-CD28/CD49D for Luminex analysis. Cells from four participants with the high IgG recognition of \( rTm\)-WAP49 were measured for cytokine production in supernatant after stimulation. 250.00 live cells were restimulated with either \( rTm\)-WAP49 or \( Tm\)-ES at 50 ug/mL and co-stimulatory molecules anti-CD28/CD49. Samples of supernatant were taken at 34, 60, 84, and 132 hours. Supernatants were measured for IL-4, IL-5, IL-13, and IFN-\( \gamma \) concentrations by Luminex analysis. Values for all four participants were averaged and compared to those of controls.
5.4 Discussion

To understand the translatability of the immunogenicity and protective efficacy observed for the *T. muris* derived immunogen *rTm*-WAP49 in the mouse model for *T. trichiura*, we evaluated the *in-vitro* antigenic cross-recognition of *rTm*-WAP49 by serum and PBMC from a population endemic for this human whipworm.

Given that whipworm infection begins early in life, we suspected that a number of participants may have had prior exposure, even if they did not have active infection at the time of enrollment. Since, there are no *T. trichiura*-specific antigens to determine prior exposure, we chose to evaluate the entire study population for their recognition of *rTm*-WAP49 in comparison to a control age-matched population from the USA with no travel history to a whipworm endemic region. Therefore, even though significantly higher IgG recognition of *rTm*-WAP49 was observed in the endemic population, relative to control USA population, it is difficult to judge this as true cross-reactivity with *T. trichiura*. Additional testing with samples from individuals with active *T. trichiura* infection alone would be ideal future direction.

Pre-existing IgE antibodies to recombinant *Na-ASP-2* acquired during natural hookworm infection led to an allergic phenotype in select patients, halting vaccine development against *Na-ASP-2* (141). In our studies evaluating for pre-formed IgE recognition of *rTm*-WAP49 by ELISA, we observed significant antigen-specific IgE in five subjects. However, in general we the mean IgE values were not significantly different between participants in the endemic area vs non-endemic controls. It is important to note that, unlike IgG, levels of IgE antibodies can significantly fluctuate in serum when exposed to parasites or allergens, leading to non-specific binding (137). One possible future evaluation would be to measure in the serum antigen-specific to total IgE ratio, which has been described as a more reliable marker of allergen-specific response (137, 139).
While we observed IgG recognition of rTm-WAP49 from Honduran participants suggest a degree of *T. trichiura* to *T. muris* cross reactivity, T cells from these individuals did not show any activation response after stimulation with rTm-WAP49. Additional attempts investigating multiple stimulation techniques that included varying activation times, concentrations of the antigen, and methods of evaluation for T cell activation were unsuccessful. Furthermore, we did not observe activation with Tm-ES. One explanation is that the frequency of anti-Tm-WAP specific T cells in the periphery are too low to respond to the in vitro stimulation protocol adopted. Future evaluations could include stimulation of PBMCs with the recombinant *T. trichiura* WAP homolog. In addition, endemic PBMCs could be used to create a humanized SCID mouse model, as shown by Taylor and Else (112), and evaluated for immune response to immunization.
Chapter VI

Conclusions and Future Directions
6.1 Overall Conclusions

Human whipworm (*Trichuris trichiura*) is pervasive intestinal parasite that causes malnutrition and inflammatory bowel disease (IBD), predominately among the world’s most impoverished billion people. Available treatment strategies have had little impact on the disease prevalence or morbidity. A vaccine against human whipworm would provide a low-cost and long-term solution, yet the identification of candidates is lacking.

The major goal of this investigation was to identify and assess protective efficacy of immunogens corresponding to *T. muris*, a mouse pathogen with significant gene and protein sequence homology to *T. trichiura*. The central hypothesis that we set out to answer in a murine model was that *Trichuris* spp. stichosome secretory proteins induce protective type 2 immunity.

To achieve this goal, we designed a systematic approach of screening *T. muris* excretory secretory (*Tm*-ES) products for identifying immunogenic proteins and testing their efficacy in the susceptible AKR mouse model. This was necessary because, unlike in the cases of hookworm or roundworm (1), there were no specific *T. trichiura* or *T. muris* candidate immunogens reported, and *Tm*-ES immunized mice exhibit significant protection (>90%) against oral *T. muris* challenge.

An immunoscreen of the *T. muris* adult cDNA expressional library with protective *Tm*-ES antisera identified several targets for determining immunogenicity and efficacy. The most abundant candidate was a whey acidic protein (*Tm*-WAP), followed by the Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 (CAP-1). BLAST analysis of the *T. muris* genome on WormBase revealed that the *Tm*-WAP is gene TMUE_s0165000300 and the *Tm*-CAP-1 is TMUE_s0030008500 (120, 121). Both these candidate genes encode proteins (WAP and CAP-1) that share a high degree of amino acid sequence identity between *T. muris* and *T. trichiura*, which suggests that key immunological epitopes may be shared between the macromolecules.
The near full-length Tm-WAP (rTm-WAP49) was expressed in P. pastoris X33 with a 6His-tag at C-terminus for IMAC purification. The most conserved repeat of Tm-WAP, fragment 8, was expressed in E. coli BL21 with a Necator americanus glutathione s-transferase-1 (Na-GST-1)-tag at the N-terminus to improve expression and solubility as a recombinant fusion protein (rTm-WAP+Na-GST-1). In addition, rNa-GST-1 is a hookworm vaccine candidate (108), and was selected among GST-tags for the potential downstream evaluation as a pan-anthelminthic vaccine candidate. Full-length recombinant Tm-CAP-1 (rTm-CAP-1) protein was expressed with an 8 his-tag at C-terminus in Escherichia coli (E. coli) BL21.

Subcutaneous immunization with rTm-WAP49 or rTm-WAP-F8+Na-GST-1 formulated with ISA 720 adjuvant led to a significant reduction in worm burden after oral T. muris challenge infection. In contrast, rTm-CAP-1, the second most highly expressed protein recognized by Tm-ES antisera, was not efficacious.

Immunization with rTm-WAP49 or rTm-WAP-F8+Na-GST-1 induced a strong TH2 response (i.e. IL-4, IL-9, and IL-13) at the parasite-draining MLNs, vaccine-draining ILNs, and the spleen. While recombinant Tm-CAP-1 did induce some TH2 response, it was significantly less effective than either rTm-WAP49 or rTm-WAP-F8+Na-GST-1. During the "critical period" of worm expulsion (days 0-21), mice immunized with either rTm-WAP49 or rTm-WAP-F8+Na-GST-1 showed significant anamnestic immunity in terms of high levels of TH2 cytokines, similar to those in mice immunized with Tm-ES. Such responses were not observed in mice immunized with rTm-CAP-1. In addition, protection correlated with IgG1 to IgG2a ratio, leading to the highest ratios by Tm-ES immunized, followed by rTm-WAP49, then rTm-WAP-F8+Na-GST-1, and finally rTm-CAP-1. Antisera from mice immunized with rTm-CAP-1 showed cross-recognition of Tm-ES antigen by ELISA, but at nearly a 100-fold less efficiently than the antisera from mice immunized with either rTm-WAP49 or rTm-WAP-F8+Na-GST-1 proteins. These results suggest that recombinant Tm-WAP is potentially a critical component of Tm-ES for inducing protection against T. muris challenge.
Since the stichosome plays a critical role in helminth pathogenicity (70, 71), and its secretions are considered the primary source of macromolecules in Tm-ES, we used serum from mice immunized with rTm-WAP49 and localized the native WAP to the stichosome of T. muris within the infected mouse cecum tissues. Immunofluorescence staining suggest WAP is secreted from the stichosome into the colonic mucosa, but further detailed exploration is necessary to understand how Tm-WAP is produced, trafficked, and ultimately secreted.

Both rTm-WAP49 and rTm-WAP-F8+Na-GST-1 proved to be significantly protective and were further evaluated for stability, purity, and homogeneity. rTm-WAP-F8+Na-GST-1 was found to be stable, with a melting temperature of 57°C by CD, homogenous with one major peak by UPLC, and with no signs of contaminants in any of the analytical assays used. We found rTm-WAP49 to have remarkable thermal stability, with a melting temperature of melting temperature of 75°C by CD, and no signs of contaminants. However, various rTm-WAP49 fractions collected by HPLC revealed protein oligomerization. Reducing rTm-WAP49 leads to a single 49 kDa protein by SDS PAGE, identifying the likely cause of oligomers as disulfide bonding. This is not surprising, as whey acidic proteins by definition have a disulfide core domain, and rTm-WAP49 has 8 of these domains in repeat. Western blot of Tm-ES with rTm-WAP49 antisera revealed multiple bands with cross recognition, suggesting that native Tm-WAP exists in a multimeric state, similar to that reported for T. trichiura WAP (21). Protein oligomerization has been noted as an advantageous feature for protein function and control (163), and thus, may be an inherent property of native WAP proteins of Trichuris spp.. Nonetheless, rTm-WAP49 may need to be re-formulated or re-engineered to create a homogenous protein.

Lastly, we assessed the translatability of the immunogenicity observed for T. muris-derived rTm-WAP49 by measuring its cross-reactivity to serum and PBMC samples from 236 patients from the human whipworm endemic region of Colomunecagua, Honduras. First, we selected and confirmed active transmission of T. trichiura by qPCR of parasite DNA extracted from stool samples. Sera from patients revealed significant IgG recognition of rTm-WAP49 when
compared to sera from non-endemic control individuals. However, given the possibility of cross-reactivity induced by other nematode infections, a more comprehensive screening will need to be performed to validate these findings. Natural infection with *T. trichiura* does not appear to generate pre-formed IgE antibodies as we did not observe their recognition of *rTm* WAP49 by ELISA. This is a significant finding as pre-formed IgE antibodies to recombinant *Na-ASP-2* were sufficient to induce generalized urticaria in vaccinated individuals, halting the vaccine’s development (141). In addition, after three immunizations in mice with either of the two recombinant WAP proteins we did not observe a significant parasite-specific IgE to total IgE ratio suggestive of the vaccine inducing the generation of IgE antibodies.

In summary, we evaluated two *Trichuris* spp. secretory proteins, *Tm-WAP* and *Tm-CAP-1* for immunogenicity and protective efficacy against *T. muris* challenge in the AKR mouse model. We found that *Tm-WAP*, expressed as two independent recombinant proteins, *rTm-WAP49* and *rTm-WAP-F8+Na-GST-1*, induced protective type 2 immunity in a murine model manifested in terms of high IgG1 to IgG2a ratio and TH2 cytokine production. While both recombinant proteins used for immunization and efficacy studies exhibited high degree of purity and stability properties assessed by a variety of analytical methods, *rTm-WAP49* has a more complex biophysical profile, with oligomerization to dimers and trimers through intermolecular disulfide bond formation. Recognition of *rTm-WAP49* by endemic patient samples suggests that conserved epitopes may exist between *T. muris* and *T. trichiura* derived WAPs. To our knowledge, this is the first study identifying a promising immunogen for further investigation of its vaccine potential against *T. muris* (105) and eventually against *T. trichiura*. 
6.2 Future Directions

The goal of this dissertation was to identify and determine the protective efficacy of immunogens from *T. muris*, the mouse homologue of the human parasite *T. trichiura*, that would aid in the understanding of immune parameters important for the control/treatment of *T. trichiura* infection.

The research presented here identified two recombinant protein derivatives of *T. muris* whey acidic protein (*Tm*-WAP) that provided partial protection against worm challenge after parenteral immunization in the AKR mouse model. While protective TH2 immune responses were detected within the mesenteric lymph nodes of mice after subcutaneous immunization, future studies testing mucosal routes of delivery (i.e. oral, sublingual, or intranasal) of the immunogens formulated in appropriate mucosal targeting adjuvants could be more effective to induce mucosal immunity relevant for this gut pathogen. Since mucosal tissues are more tolerant for induction of immune responses, immunogens targeted for mucosal tissues need to be formulated with the appropriate adjuvants. The most common adjuvants used are bacterial toxin (wild type or mutated) and others include those that effectively modulate innate immune mediators to aid in promoting adoptive immune responses. One such adjuvant used in our group has been the synthetic glycolipid alpha-galactosylceramide (aGalCer) that activates natural killer T cells (NKT) to induce maturation of dendritic cells and promote efficient antigen presentation for enhanced T and B cell responses.

Our data suggests that *Tm*-WAP is directly secreted from the stichosome into the surrounding colonic mucosa, yet its function is not known. Since WAP homologues within *T. trichiura* (Tt52 and Tt95) have been recognized for their ability to generate pores in a planar lipid bilayer (21–23), it would be important to evaluate whether *Tm*-WAP exhibits pore-formation function and whether *Tm*-WAP antisera can neutralize this bioactivity.
Our research shows that the recombinant near full-length Tm-WAP induces potent TH2 responses at levels similar to that induced by Tm-ES in key secondary lymphoid tissues, yet, Tm-ES elicits near complete protection relative to 40% worm burden reduction by rTm-WAP49. To better explore immunization-induced protection, a number of physiological mechanisms that are reported to facilitate T. muris expulsion can be explored, such as granulocyte secretion of neutralizing proteins and epithelial cell hyperproliferation and turnover (4). Perhaps the most important of these physiological mechanisms to evaluate would be intestinal goblet cell secretion of mucins and resistin-like molecule-β (RELMβ), largely driven by IL-4, IL-13, and IL-22, which block worm motility and epithelial attachment (4).

An aim of this dissertation was to evaluate human recognition of Tm-WAP. While T. trichiura is specific to humans, it may be possible to adapt the helminth to a humanized mouse model. Work by Taylor and Else demonstrated that severe combined immunodeficient mice infused with endemic human PBMCs could develop an immune response to vaccination with T. muris ES and homogenate, suggesting it may be adaptable to a vaccine animal model.

Through protein characterization, we observed that rTm-WAP49 exists in a multimeric state. To determine whether this has any role on immunogenicity or efficacy, rTm-WAP49 could be reduced through co-formulation with cysteine amino acids. Proteins reduced in this manner have been shown to release their intermolecular (between monomers) disulfide bonding without comprising protein stability or functional epitopes (5, 6).

This thesis represents an important, but early step towards the identification of whipworm immunogens that may have utility as future vaccine candidates. While Tm-WAP identified in our studies seem to be a promising immunogen capable of inducing strong TH2 immunity, only partial protection was achieved in the mouse model employed. Thus, despite the immunodominance of Tm-WAP, there may be more antigens that synergistically augment protection through co-immunization. Therefore, it is important to investigate which of the other macromolecules within
the *T. muris* ES that may be important along with *Tm*-WAP to induce the sterile immunity seen with *Tm*-ES immunization.
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Vita

Neima Briggs was born in Austin, Texas, the son of Samileh Mozafari and Benjamin Briggs. Following his graduation in 2008 from Westwood High School, Austin, Texas, Neima entered the at the University of Texas at Austin. In May 2011, he completed his studies within the Dean’s Scholars Honors Program with a Bachelor of Science in Biology with Special Departmental Honors from the College of Natural Sciences. Between 2011 and 2012, Neima served as a United States Fulbright Scholar at the Center for Public Health Research in Valencia, Spain. In July 2012, Neima began his MD/PhD studies at the University of Texas Health Science Center at Houston.

The long-standing disregard of certain diseases became apparent to Neima as a first-year medical student on medical brigade to Honduras, compelling him to seek a research topic that addressed these disparities in global health. Through the co-advisement of Dr. Jagannadha Sastry at MD Anderson UTHealth GSBS and Dr. Peter Hotez at the National School of Tropical Medicine of Baylor College of Medicine, Neima forged a dissertation, presented here, that sought to identify protective immunogens that may aid in the development of a whipworm vaccine.

During his graduate work, Neima has received numerous awards, including the ASTMH Benjamin H. Kean Travel Fellow in Tropical Medicine and the MD Anderson UTHealth GSBS Grady Saunders “Investing in Students Futures” Scholarship. Neima also received advanced training in tropical medicine and received his Diploma of Tropical Medicine from Baylor College of Medicine. Neima will graduate from the MD/PhD Program in May 2019, with plans to pursue a career as a physician scientist. His goal is to develop novel approaches in the eradication of the infectious diseases that plague underserved populations.

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