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HELMINTHS AND IMMUNITY AGAINST TUBERCULOSIS

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Stockholm 2006

Published and printed by Karolinska University Press
Box 200, SE-171 77, Stockholm, Sweden

ISBN: 91-7357-035-4

1 ABSTRACT

The efficacy of Bacille Calmette Guerin (BCG) is low and incidence of tuberculosis (TB) is high in those areas where helminths are endemic. Protection against tuberculosis requires strong cell mediated immunity while chronic helminth infections induce responses characterized by dominant Th2 responses as well as up-regulated regulatory T cell activity. Such immunomodulation caused by helminths were shown to result in hyporesponsiveness against unrelated antigens. We hypothesized that chronic helminth infection could affect the ability of the host to control mycobacterial infections and/or the efficacy of vaccination against TB.

This thesis is based on five separate clinical and experimental studies. The first was aimed to assess whether intestinal helminth infections could affect TB specific cellular responses in individuals with prior mycobacterial exposure as well as to investigate the impact of worms on the immunogenicity of BCG vaccination in humans. The result indicates that treatment of intestinal worms results in significant improvement in mycobaterial antigen induced T-cell proliferation and IFN-γ production. Moreover, vaccination with BCG significantly improves PPD specific cellular responses in treated individuals but not in the untreated controls.

The second study examined whether the helminth associated down modulation in cellular immune responses against TB antigens is a reflection of altered immunity against mycobacterial infections. We assessed this using a mouse model where mice were infected with *Schistosoma mansoni* and later challenged with *M. bovis* BCG. Later analysis of bacterial loads demonstrated that worms could impair the ability of the animals to contain *M. bovis* BCG infection. This was associated with low Th1 responses and enhanced Th2 responses.

In the third study we assessed if worm associated reduction in the immunogenicity of BCG observed in the first human study may suggest altered efficacy of BCG vaccination against *M. tuberculosis*. We infected C57Bl/6 mice with *S. mansoni* and later vaccinated them with BCG. Eight weeks post vaccination; the animals were challenged with *M. tuberculosis*. Comparison was then made between animals BCG vaccinated in the presence of *S. mansoni* infection and controls with regard to the load of *M. tuberculosis*, lung pathology as well as *in vitro* cellular responses to TB antigens. The result shows that there were significantly higher bacterial loads in the *Schistosoma* infected group compared to controls. Histological evaluation showed that the lungs of *Schistosoma* pre-infected mice had more pathologic changes compared to controls. Moreover, *in vitro* nitric oxide and IFN-γ production were markedly lower in the *Schistosoma* infected group suggesting that helminths could negatively influence the efficacy of BCG.

In the fourth study we examined whether reduction in the *in vitro* cellular responses to TB antigens associated with worm infection in humans (study I) as well as the poor immunity against mycobacterial infections observed in animal models (studies II) could support the presence of a link between intestinal helminths and clinical TB. We conducted a case-control study and showed strong association between helminths and active TB.

In the last paper we examined the impact of deworming of helminth infected populations on the immunogenicity of BCG vaccination and the mechanisms behind helminth induced immunomodulation that affects immunogenicity of BCG. We recruited volunteers with asymptomatic helminth infection and with no sign of prior infection with mycobacteria. The subjects were randomized to anti-helminthic therapy or placebo treatment. The volunteers were later BCG vaccinated and *in vitro* immunological analysis was conducted. The result showed that indeed worms do affect the immunogenicity of BCG and this was associated with reduced Th1 responses, high TGF-β secretion but not enhanced Th2 responses.

In conclusion, our results demonstrate that immunomodulation by helminths affects immune responses against TB as well as the immunogenicity of BCG. This is a finding with practical consequences as treatment against helminth could offer a novel means to reduce the burden of TB in areas where worms are endemic.

Key words: tuberculosis, BCG, helminths, immunomodulation, vaccination

2 LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to by their roman numerals

I **Elias D**, Wolday D, Akuffo H, Petros B, Bronner U, Britton S. Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after BCG vaccination. *Clin Exp Immunol* 2001 Feb; 123(2): 219-25.

II **Elias D**, Akuffo H, Thors C, Pawlowski A, Britton S. Low dose chronic S. mansoni infection increases susceptibility to Mycobacterium bovis BCG infection in mice. *Clin Exp Immunol* 2005 Mar; 139(3):398-04.

III **Elias D**, Akuffo H, Pawlowski A, Haile M, Schon T, Britton S. Schistosoma mansoni infection reduces the protective efficacy of BCG vaccination against virulent Mycobacterium tuberculosis. *Vaccine* 2005 Feb 23(11):1326-34.

IV **Elias D**, Mengistu G, Akuffo H, Britton S. Are intestinal helminths risk factors for developing active disease? *Trop Med Int Health* 2006 Apr 11(4):551-8.

V **Elias D**, Akuffo H, Aseffa A, Engers H, Britton S. Poor BCG immunogenicity in helminth infected population is associated with enhanced TGF-β production. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
BCG	Bacille Calmette Guerin
CD	Cluster of differentiation
CFU	Colony forming unit
CNS	Central nervous system
ConA	Concanavalin A
EAE	Experimental autoimmune encephalitis
ELISA	Enzyme linked immunosorbant assay
ELIspot	Enzyme linked immunospot
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IL-10	Interleukin 10
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
iNOS	Inducible nitric oxide synthase
MVA	Modified virus ankara
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemaglutinin
PPD	Purified protein derivative
SHIV	Simian immunodeficiency virus
TB	Tuberculosis
TGF-β	Transforming growth factor beta
Th1	T helper one
Th2	T helper two
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
Tregs	Regulatory T cells
DMSO	Dimethylsulphoxide

1 INTRODUCTION

1.1 TUBERCULOSIS

Tuberculosis is a bacterial disease caused by *Mycobacterium tuberculosis* which is spread through aerosols from patients with pulmonary disease.

Overall a third of the world population is infected with *M. tuberculosis* (1) but the vast majority remain without symptom. Only 5-10% of the infected will progress to develop clinical tuberculosis. The proportion of people who go on to develop the disease increases in the presence of factors that cause immunosuppression including diabetes, alcoholism, malnutrition, stress and above all co-infection with HIV. *M. tuberculosis* infected HIV positive subject runs 10% annual risk of developing active tuberculosis as opposed to 10% life time risk in HIV negative individuals (2).

1.1.1 Distribution

TB has a worldwide distribution, however as it is the case with most infectious diseases (Fig.1), the bulk of the problem concentrates in low-income countries in south-east Asia and sub-Saharan Africa (3). Globally over 8 million new cases of active TB and 3 million TB deaths occur annually. Ninety five percent of all TB deaths occur in south-east Asia and sub-Saharan Africa (1).

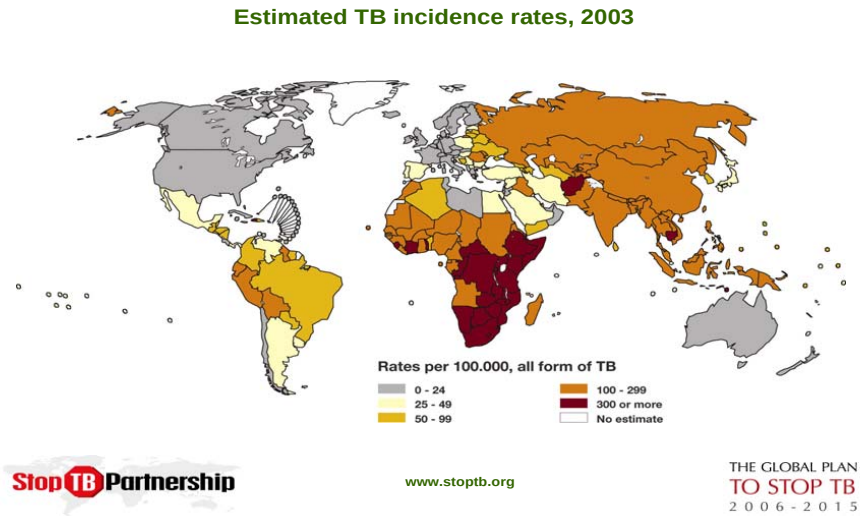


Fig 1 Estimated worldwide TB incidence rates from data collected in 2000. The numbers show the estimated incidence of TB cases for each region.

The global burden of tuberculosis remains enormous, mainly because of poor control in Southeast Asia, sub-Saharan Africa and Eastern Europe, the emergence of multidrug resistant strains as well as the high rates of *M. tuberculosis* and HIV co-infection in countries south of Sahara and Asia (1).

3.1.2 Immunity against TB

M. tuberculosis usually enters the host via inhalation of droplets containing viable bacteria. The bacterium reaches alveolar spaces and is ingested by macrophages and dendritic cells (4). Infected cells enter draining lymph nodes where antigen specific T cells are stimulated. This results in the induction of inflammatory responses with the consequent development of granulomatous lesions where different T cell populations participate in protective immune responses (5). The granuloma is surrounded by a fibrotic wall which separates it from the surrounding tissue (6).

Studies conducted both in animal models and in humans suggest that CD4 T cells play critical role, the effector function of which is mainly mediated by the production of interferon gamma (IFN-γ). Genetic defects in pathways leading to IFN- γ production are associated with extreme susceptibility to tuberculosis (7-12). IFN-γ is the central mediator of macrophage activation with the resulting increase in molecules required for mycobacterial killing, and is also involved in activating antigen-presenting functions. While CD4 T cells are an important source of IFN-γ, other T cell populations were also shown to produce IFN-γ. Such cells include CD8 T cells as well as γδ T cells that are restricted by unconventional presentation molecules (CD1).

CD8 T cells recognize antigenic peptides in the context of MHC class I and are thought to contribute to immune responses against TB via cytotoxic activity (13) and IFN-γ production (14,15). γδ T cells recognize non-peptide antigens in the context of CD1 molecules and were shown to be involved in *M. tuberculosis* induced immune responses (16). The exact role of γδ T cells in TB immunology is not clearly defined but are thought to contribute by cytokine production in the early phases of infection which is thought to provide help to the antigen presenting cells and were also shown to be involved in cytotoxic functions (17). Although IFN-γ is crucial for protection against tuberculosis, protection is not directly associated with the magnitude of IFN-γ responses (18,19)

Studies indicate that the CD4 T cell population includes both effector and regulatory subsets (20). The regulatory T cells are normally associated with down-regulation of the immune response and were initially thought to be solely involved in inhibiting pathogenic and auto reactive T cells (21,22) however their involvement during infections is becoming more and more clear (23) and evidence is accumulating that the outcome of primary infection with *M. tuberculosis* is influenced by the balance between these two subsets of CD4 T cells (22).

3.1.3 Development of vaccines against TB

The most efficient way to control TB would be the use of effective vaccines. The current vaccine (BCG), an attenuated strain of *Mycobacterium bovis,* was developed in the 1920's by two French scientists (Albert Calmette and Camille Guerin). This vaccine provides good protection against childhood tuberculosis (24) but fails to show consistent efficacy against adult tuberculosis (25,26). This coupled with the rising TB epidemic worldwide has compelled WHO to declare TB as a global emergency (27). This has prompted global effort to control the disease and to develop a vaccine more efficacious than the one currently in use. Approaches to develop such a vaccine have included the use of live attenuated bacteria, recombinant bacteria, subunit vaccines and DNA vaccines. Out of these viable recombinant bacteria and subunit vaccines are well developed and have entered clinical trials (6).

3.1.3.1 Viable vaccines

There are two vaccines under this category which have entered into clinical trial. This include the BCG30 and rBCG that expresses listeriolysin and made to be urease deficient denoted by ureC hly+ rBCG. The recombinant BCG30 is constructed to over express an immunodominant antigen (Ag85), an antigen shared by both *M. tuberculosis* and BCG. The construction of the later (ureC hly+ rBCG) allows antigens to leak into the cytoplasm for presentation via the MHC-I pathway. Both were shown to provide more protection than BCG in animal models (28,29) and are under investigation in clinical setup.

3.1.3.2 Subunit vaccines

Subunit vaccines are basically designed to be used as a booster vaccine following initial vaccination with live vaccine. Three subunit vaccines have entered clinical

trials in the last 2 years. These include: *M. tuberculosis* 72F, Fusion protein comprising of ESAT-6 and Ag85B and modified virus Ankara (MVA). The first two are fusion protein while the later is a replication deficient *Vaccinia* virus expressing Ag85A. All the three have demonstrated good protection in animal models and have progressed into phase I clinical trial (30-33).

3.2 HELMINTHS

3.2.1 Epidemiology

Helminths, a name given to a group of multicellular worm parasites including nematodes, cestodes and trematodes, remain among the most common diseases of man affecting up to 25% of the world's population (34). Intestinal helminths account for up to 40% of total disease burden among children in low-income countries (35). Of particular worldwide importance are the round worms (*Ascaris lumbricoides*), whipworms (*Trichuris Trichiura*), hookworms (*Necator americanus* or *Ancylostoma duodenale*), members of the genus Schistosoma (*S. mansoni*, *S. haematobium*, *S. japonicum*), *Strongloides stercoralis* or the filarial helminths (34). Infection with parasitic helminths occurs by ingestion of eggs as in *A. lumbricoides*, *T. trichiura*, by direct penetration of skin by infective larvae (hookworms, *Schistosoma* spp or *Strongloides stercoralis*) or through the bite of infected insect vectors (Filariasis) (36). Most helminth parasites have complex life cycles (Fig 2) consisting of intermediate stages that require different intermediate hosts to complete their life cycle.

3.2.2 Pathogenesis and clinical manifestations

A wide spectrum of disease occurs following infection with parasitic helminths. Most of the burden is a result of chronic intestinal infection although those preferring to dwell in the tissue cause other illness as diverse as elephantiasis a consequence of lymphatic filariasis or acute dermatitis caused by migrating larvae of hookworms or *Strongloides* spp. Larva migration may also cause eosinophilic pneumonitis.

Anemia is commonly caused by helminths, primarily by hookworm infection. These parasites also cause varying degrees of malnutrition (37). Intestinal worms may impair appetite possibly due to inflammatory enteritis mediated by host immune response (36). Ascaris and hookworms also secrete potent inhibitors of pancreatic enzymes affecting digestion and absorption of nutrients in the intestine (38).

3.2.3 Strategies for control

Control of parasitic helminths is mostly based on mass treatment programs. Regular treatment with broad-spectrum anti-helminthic drugs (benzimidazole or albendazole) are good at reducing morbidity from soil transmitted helminths (36) while ivermectin works well in areas endemic for filarial diseases especially onchocerciasis (39). Other control methods such as the use of vaccines is theoretically attractive but remain largely elusive due to the lack of good animal models, poor understanding of the mechanisms that allow the parasite survive in the host for years and the poor understanding of the immune mechanisms of control. However, hookworm vaccine consisting of recombinant larval antigen ASP2 is found to be effective in animal models and is undergoing clinical development in humans (40).

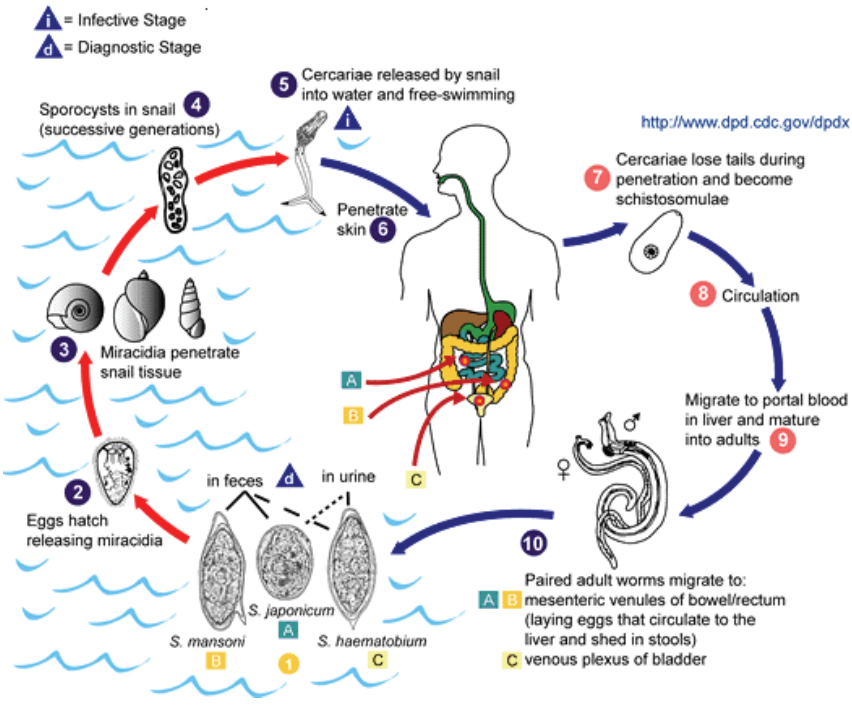


Fig.2 Life cycle of *Schistosoma spp.* (adapted from http:/www.dpd.cdc.gov/dpdx)

3.2.4 Immunological impact of helminths

Helminths are long-lived multicellular parasites, which induce strong and directed immune responses in their hosts. Common features of helminth infections include slow

accumulation of parasite burdens, the prevalence of chronic infection, and the primary role of the immune response in pathology (41). The following are the major immunological impact of chronic helminth infections.

3.2.5 The Th1/Th2 shift

Helminth infections are potent inducers of Th2 type responses characterized by eosinophilia, high titers of circulating IgE as well as increased secretion of interleukin 4 (IL-4), IL-5 and reduced Th1 type responses (42). Early work showed that helminth infection or immunization by worm antigens amplifies Th2 response to bystander antigens (43). These have been demonstrated in the mouse model (44-46) and were also demonstrated in humans (47-50). The enhanced Th2 activity is usually (not always) associated with impaired antigen specific and non-specific Th1 responses.

3.2.6 Enhanced Treg activity

It is becoming increasingly clear that helminth infections, in addition to stimulating vigorous Th2 responses, induce suppressive T cell populations known as regulatory T cells (Tregs) (23). Tregs produce inhibitory cytokines (IL-10, TGF-ß) that suppress inflammatory responses. They are also known to interfere with effector T cell activation in a contact dependent manner (55).

Several investigators showed that worm antigen specific T cell anergy is common in infected individuals and that their reactivity can be regained in the presence of antibodies that neutralize regulatory cytokines IL-10 or TGF-β (51-54). This was reported to be the evidence for increased regulatory T cell activity during helminth infection.

Such changes in the immune response induced by chronic helminth infections may have an impact on the host's ability to cope with other unrelated infections such as that due to *M. tuberculosis*.

3.2.7 Impact of helminths on other diseases

Several investigators have shown that immune profile induced by a given infection could have an impact on the outcome of subsequent infections or vaccinations (56,57). Indeed series of studies conducted both in the mouse model as well as in humans produced evidences consistent with this hypothesis (18,45,58-61).

Some important clinical conditions suggested to be influenced by helminth infections include:

3.2.7.1 HIV/AIDS

Reports have shown that cells obtained from helminth infected individuals were more susceptible to HIV-1 infection *in vitro* compared to cells from uninfected controls (62,63). It has also been reported that deworming of HIV+ individuals with heavy worm loads is associated with reduction in plasma viral load (64). A recent report from Kenya indicated that mother-to-child transmission of HIV-1 was found to be enhanced by infection of the mother with helminths (65)**.** Using primate model, Chenine et al showed that *S. mansoni* infection increases the plasma viral load of Semian HIV (SHIV) in rhesus macaques (66) and this was accompanied by a Th2 shift in immune profile. However, the rate of progression of HIV disease is not different in populations living in helminth endemic parts of the world compared to the western world where such infections are almost non-existent (67). Indeed a study conducted in Ethiopia (endemic for helminths) indicated that the rate of CD4 T cell decline is slower in HIV+ Ethiopians compared to their Dutch counterparts(68). Brown and colleagues have shown that treatment of *S. mansoni* infection in HIV+ cohorts in Uganda increases plasma viral loads (69). Therefore it is not clear whether the observations made *in vitro* or in the animal model could have relevance in clinical situation.

3.2.7.2 Mycobacterial infections

A series of studies conducted both in the mouse model as well as in humans produced evidences supporting an impact of helminths on immune responses against mycobacterial infections (18,45,58-61). Peripheral T cells obtained from persons infected with filarial parasites or *Schistosoma* spp were shown to respond poorly to parasite antigens (70) as well as to unrelated antigen mixture obtained from *M. tuberculosis* (71). Bentwich and colleagues suggested that such helminth associated hyporesponsiveness is a result of chronic immune activation (72). We have demonstrated in several studies that helminths affect responses against TB antigens or the outcome of vaccinations (18,45,59,73-75). Moreover some investigators have shown in a clinical setting that helminth infections may be associated with increased incidence of mycobacterial diseases. Diniz and colleagues demonstrated that worm infected leprosy patients are more likely to be multibacillary compared to helminth

free controls (61) while Elias et al and Tristao-Sa et al showed an association between active pulmonary TB and helminths (60,75). However such association studies do not confirm cause-effect relationship.

3.2.7.3 Autoimmune diseases

Work by Sewell *et al* showed that treatment of mice with *S. mansoni* ova reduces the degree of muscle paralysis and inflammatory cell infiltration into CNS in experimental autoimmune diseases (EAE) at the same time polarizing immune profile towards Th2 type (76,77). Infection of non obese diabetic mice with *S. mansoni* was shown to reduce the risk of autoimmune disease by 50% (78). In inflammatory bowel disease was ameliorated using *Trichuris suis* in clinical setup (79). This suggests the possibility of a beneficial effect of carrying worms in reducing the severity of autoimmune disorders.

3.2.7.4 Allergic disorders

Evidence is accumulating that chronic helminth infections are inversely associated with immune disorders (41). A recent study conducted on school children in Gabon demonstrated negative correlation between helminths infection and skin prick test responses to allergens (80). Sellassie and colleagues showed that Ascaris and/or hookworm infection show protective effect on the prevalence of Asthma (81).

It is plausible to think that most of the infections common in low income settings have been with humanity for several millennia and there appears to be co-evolution between these infections and the body's immunity and it is likely that the interaction may have beneficial consequences for both the host and parasite . The sudden change in the environment as in the western society could offset the balance and may result in immune disorders which may be manifested in the form of autoimmune diseases and/or allergic manifestations.

Most autoimmune disorders arise as a result of Th1 responses directed against self tissues while allergic disorders are mediated by Th2 type immunity. It therefore appears paradoxical that both Th1 and Th2 mediated diseases are low in those areas where helminth infections are common (41). Some authors argue that this may be due to competition between responses to chronic infections (notably helminths) and allergens for growth factors or cytokines or for suitable niches within the lymphoid

tissue (reviewed in (41)). It could as well be that down regulation due to sustained immune challenge may affect immune disorders. Continuous stimulation may cause receptor down modulation or overt suppression through regulatory T cell population. Such regulatory mechanisms may intercede against Th1 mediated inflammatory diseases or Th2 mediated allergic disorders.

4 OBJECTIVES

The work leading to this thesis was undertaken to investigate the impact of chronic helminth infection on immunity against tuberculosis and on the efficacy of BCG vaccination.

The specific objectives were:

- To investigate whether deworming could affect responses against mycobacterial antigens
- To evaluate whether helminth infection affects the immunogenicity of BCG vaccination
- To assess the impact of helminths on resistance against mycobacterial infection in animal model
- To investigate the impact of worm infection on the efficacy of BCG vaccination in the mouse model
- To examine the association between intestinal helminth infection and active TB in clinical set-up

5 MATERIALS AND METHODS

5.1 STUDY PARTICIPANTS AND MATERIALS

Paper I was based on materials obtained from college students from Addis Ababa, Ethiopia (N=64) with helminth infection. They were screened for prior mycobacterial infection using the Mantoux test and those without evidence of prior mycobacterial infection were BCG vaccinated and later response of their peripheral blood mononuclear cells to MTB antigen was analysed.

Paper II and III were based on experiments conducted in the mouse model using BALB/c and C57bl mice respectively.

In paper IV, we used materials obtained from 230 smear positive TB patients in the outpatient department of the Gondar University Hospital and their healthy household contacts (N=510). Samples obtained include stool for parasitological examination, sputum for staining for acid fast bacilli as well as blood for serological analysis of HIV infection.

Paper V involved individuals with asymptomatic helminth infection, no sign of prior mycobacterial infection i.e. no response to the tuberculin skin test (N=111).

5.2 TUBERCULIN SKIN TESTING AND BCG VACCINATION

Tuberculin PPD (2 Tuberculin units; Statens Serum Institute, Denmark) was injected intradermally on the ventral aspect of the forearm, the diameter of skin induration was measured 48 hours later following the guidelines specified in the WHO standard tuberculin test technical guide (82). BCG vaccine was injected intradermally in the left deltoid region to tuberculin negative subjects.

5.3 PARASITOLOGICAL EXAMINATION

Stool samples obtained from the volunteers were examined using the direct microscopy as well as the formol-ether concentration techniques (study I and IV) or using the Kato-katz technique (83) (study V).

5.4 PERIPHERAL BLOOD MONONUCLEAR CELL SEPARATION AND STORAGE

Heparinized venous blood was collected and PBMC isolated using the Ficoll-Hypague density gradient centrifugation. The cells were then resuspended in 10%

DMSO in fetal calf serum, aliquoted into Nunc^{TM} tubes (Cole-Parmer Inc, Illinois, USA) and frozen to -70^{o}c over night and transferred to liquid nitrogen until assays were performed.

5.5 CELL PROLIFERATION

PBMC were cultured in a round-bottom 96-well microtitre plate. The cultures were stimulated with a mitogen (phytohemaglutinin or concanavalin A) for 3 days or with purified protein derivative of *M. tuberculosis* (PPD) at a concentration of 5ug/ml for 5 days at 37^{o}C in CO_2 incubator.

5.6 CYTOKINE ELISA

Cytokine levels in cell culture supernatants were measured employing standard ELISA protocols using commercially available pair of monoclonal antibodies (R& D systems, London, UK) according to manufacturer's instructions.

5.7 ELISPOT ASSAY

In vitro IFN-γ, IL-12, IL-4, IL-5 and TGF-β secretion were also measured using ELISpot assay where PBMC were mixed with antigens at a concentration of 5ug/ml or a mitogen at a concentration of 3ug/ml and plated in duplicates of 1.5x10^{5} cells/well. After 20 hours of incubation (37^{o}C, 5% CO2 with 95% humidity), spots were visualized, counted using an ELIspot reader (AID, GmBH, Strasburg, Germany) and reported as spot forming units (SFU)/1.5x10^{5} cells.

5.8 INFECTION OF MICE WITH *S. MANSONI*

Infective larvae (Puerto Rican strain) were obtained from infected snails and mice were anesthetized by intraperitonial injection of 100ul of 8.6 mg/ml pentobarbitalnatrium and 100ul of tap water containing 30 infective larvae was placed on shaved skin area on the abdomen and left for 20 minutes for active infection to occur. The presence of the infection was checked repeatedly by direct microscopy of stool samples during the course of the experiments.

5.9 ANALYSIS OF MYCOBACTERIAL LOADS

Bacterial loads in the organs of infected mice were evaluated as described by sacrificing animals and aseptically removing their organs which were then homogenized and diluted and later plated on middle brook 7H11 containing 0.3mg/ml

polymyxin B and 5mg/ml amphotericin B. Plates were incubated at 37^{o}C and 5% CO_2 until visible colonies were observed usually 3-4 weeks after plating.

5.10 HISTOPATHOLOGY

Lung tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections with 5um thickness were cut using a microtome and stained with hematoxylin and eosin. The sections were examined blind by a pathologist experienced in the histopathology of mycobacterial diseases without prior knowledge of the experimental groups.

5.11 NITRITE ASSAY

Nitrite levels in culture supernatants were determined by the method of Green et al using the Greiss reagent(84). The titer was determined by standard curve generated by the absorbance of serial dilutions of $NaNO_2$ (Wako, Stockholm, Sweden).

5.12 ETHICAL PERMISSIONS

All work reported in this thesis have received ethical permits from the regional ethics committee of the Karolinska Institutet as well as the national ethics review board in Ethiopia. Accordingly data reported in papers I, IV and V were conducted after ethical permission from the Karolinska Institutet reference number: 01-324 and Ethiopia RDHE/71-26/99, RDHE/81-49/2003. Data reported in Papers II and III had ethical permission from the ethical committee for animal experimentation in Stockholm, reference number 48/2001-54.

5.13 STATISTICAL ANALYSES

Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL), STATA version 7 (STATA Corporation, College station, TX, USA). Data were expressed as means and standard error of mean and student's t-test was used to compare the groups in papers I and II and part of the data in paper III. CFU data were assessed by Student-Newman-Keuls test in paper III. In paper IV the association between the different variables and having active TB was evaluated using conditional logistic regression and the difference in helminth infection rate between the groups was examined using the x^2 test.

6 RESULTS AND DISCUSSION

6.1 Deworming enhances TB antigen specific in vitro cellular responses (Paper I)

The efficacy of BCG is low and the incidence of TB is highest in the tropics (25,85) where intestinal helminths are endemic (34,86). Several investigators have reported that chronic helminth infection could affect immune responses to unrelated antigens or pathogens (48-50,71,72,87-94). We therefore hypothesized that immunomodulation by intestinal helminths could provide part of the explanation why BCG performs so badly and why the incidence of TB is the highest in the tropics.

Apparently health volunteers were examined for intestinal worm infection and worm positive subjects were grouped randomly into groups. One group was given anti-helminthic therapy the other was provided with placebo. Eight weeks after anti-helminthic therapy/or placebo, cellular responses to TB antigens (T cell proliferation and IFN-γ production) were measured. Twenty-nine donors from individuals who received anti-helminthic therapy and controls were investigated for T cell proliferation and 15 for IFN-γ production. The result shows that proliferative and IFN-γ responses were significantly higher in the treated group compared to untreated controls (Fig 2). With the assay system we used in this study, levels of Th2 cytokines were not detectable in both the treated and untreated volunteers.

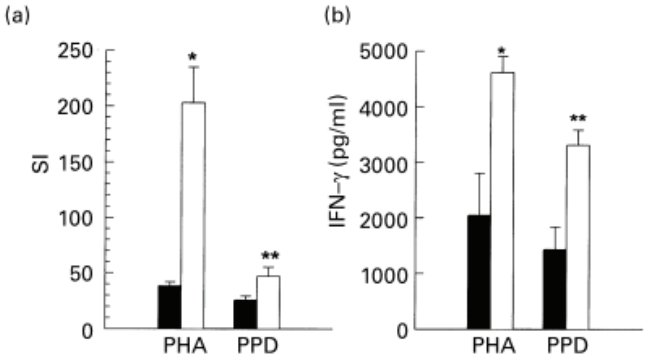


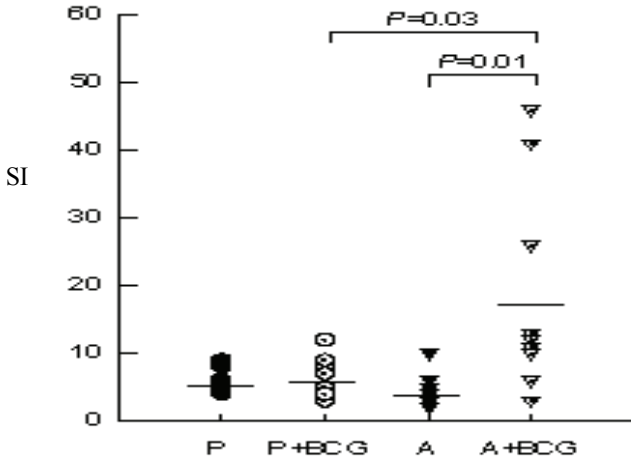
Fig. 3. Effect of anti-helminthic treatment on *in vitro* T cell proliferation (a) and IFN-γ production (b) to mycobacterial antigen mixture PPD. Peripheral mononuclear cells were obtained 8 weeks post treatment and were stimulated with PPD or a T cell mitogen (phytohaemaglutinin, PHA). IFN-γ levels were measured from supernatants of cells stimulated with PPD, PHA or culture medium alone using ELISA (* $P<0.05$, ** $P<0.01$).

The results show that chronic helminth infection impairs Th1 type responses to TB antigens and this effect could be reversed by anti-helminthic therapy. This may suggest that there is functional alteration in T cells from helminth infected subjects and this may predispose to developing active disease as effective immune response against mycobacteria is thought to be mediated by cooperation between T cells and macrophages (95) and this interaction is mediated through the production of IFN-γ (96). However it is not clear how much IFN-γ is required for effective macrophage activation nor whether the reduction in Th1 response is sufficient to make the host more permissive to mycobacterial diseases.

6.2 The immunogenicity of BCG is higher after deworming in helminth infected volunteers (Paper I).

To investigate whether intestinal helminth infections could affect the immunogenicity of BCG vaccination, 20 tuberculin skin test negative subjects in both helminth treated and control subjects were given BCG vaccination and PPD specific responses (T cell proliferation and IFN-γ) were measured prior to and 8 weeks after vaccination. The result shows that PPD specific T cell proliferation and the production of IFN-γ were markedly improved after BCG vaccination in individuals vaccinated after deworming. On the other hand the difference between pre and post-vaccination was not significant in the untreated group. Although the number of subjects included in the study was limited the finding indicated that BCG vaccination sensitizes individuals without worms better than it does in those with helminth infection (Fig. 4). While immune response against chronic helminth infection is characterized to be predominantly Th2 type and enhanced suppressor T cell activity (97), immunity against intracellular pathogens such as *M. tuberculosis* require Th1 type responses. Since Th2 as well as suppressor T cells inhibit the initiation and development of Th1 immunity it is likely that Th2/suppressor T cell activity induced by helminth infection could impair responses against TB or the immunogenicity of BCG in helminth infected populations.

(a)



(b)

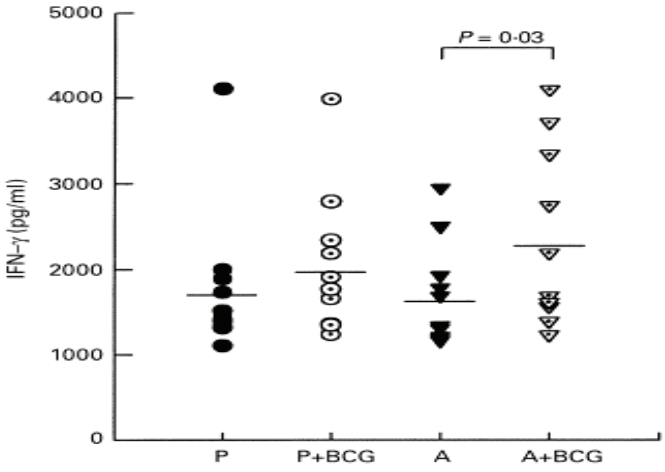
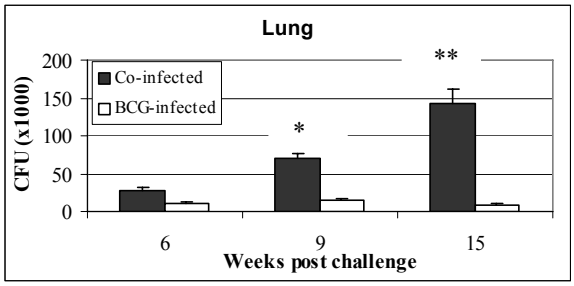


Fig 4 T cell proliferation (a) and IFN-γ responses (b) to PPD in subjects vaccinated with BCG after albendazole treatment (A) and the placebo group (P). Responses were measured at the time of vaccination and 8 weeks after vaccination. P and A are responses prior to vaccination in the control and dewormed group respectively while P+BCG and A+BCG are responses after BCG vaccination.

6.3 *S. mansoni* infection impairs resistance against mycobacterial infection (Paper II)

In paper I we observed that helminth infected individuals showed poor cellular responses to TB antigens compared to treated controls. Whether this reduced ability to mount cellular responses to TB antigen is a sign of increased susceptibility to TB was not clear. To examine this, we used the mouse model and infected the animals with *S. mansoni* and later challenged them with *M. bovis* BCG. We then followed the kinetics of bacterial growth in the lungs, liver as well as in the spleen. The result showed that indeed the *S. mansoni* infected group of animals showed higher bacterial load in the lungs, liver and spleen compared to controls (Fig.5). This finding is consistent with the findings of Actor and colleagues who demonstrated that *S. mansoni* infection in the mouse model impairs the host's ability to control *Vaccinia* virus infection which was associated with poor IFN-γ as well as IL-2 responses (98,99).

(a)



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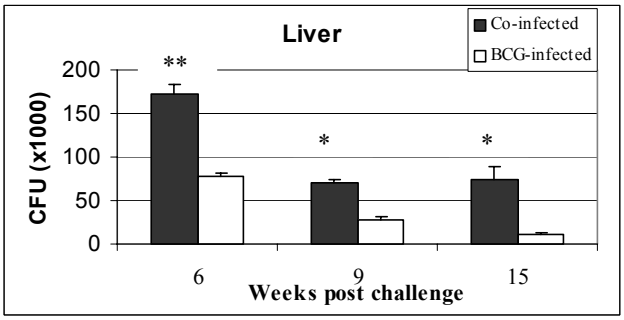

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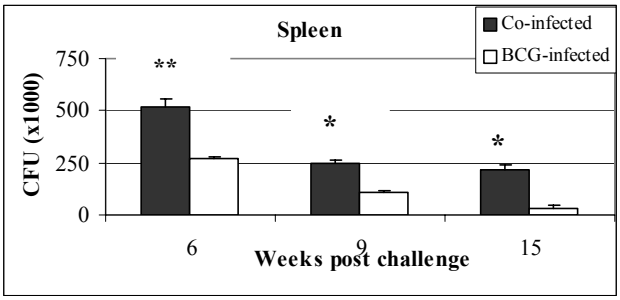
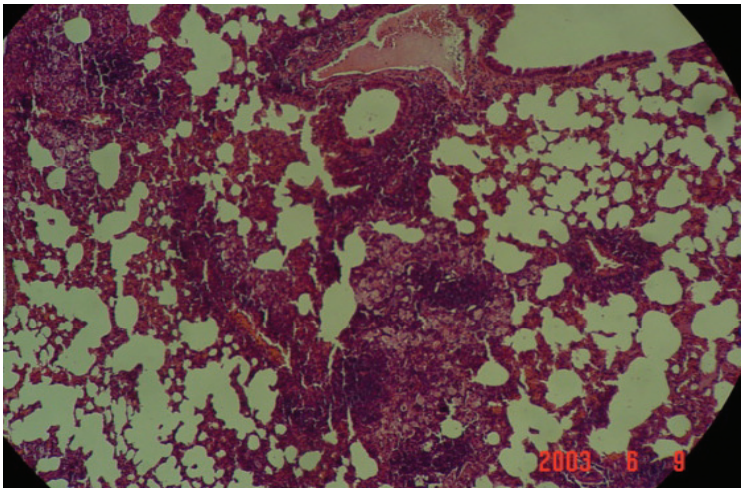


Fig 5 Bacterial loads in the lungs (A), liver (B) and spleen of *S. mansoni* pre-infected versus control mice. Ten animals were used per group to assess CFU at all time points and the experiment was repeated 3 times with comparable result. *p<o.o5, **p<0.01.

6.4 *M. bovis* BCG infection causes more lung tissue damage in *S. mansoni* pre-infected mice (paper II)

Although the model we used did not allow us to measure mortality following mycobacterial challenge, we assessed whether the high bacterial load observed in *S. mansoni* pre-infected mice cause more damage in the lungs. To do this we compared the levels of pathology in the lungs of mice infected with *S. mansoni* compared to controls. The result indicated that the lungs of co-infected mice displayed significant pathological changes that involved about 40% of the lung tissue. In the control mice however, the lungs were predominantly normal with mild lymphocyte infiltration and inflammation and the pathological changes involved about 10% of the lung tissue (Fig 6), suggesting that *S. mansoni* infection by impairing host resistance against mycobacterial infection do increase pathology in the lungs of infected animals. It has been hypothesized that one way by which mycobacteria succeed in causing disease is by inducing IL-4 responses in the host (100) which impairs antimycobacterial effector responses and enhance the toxicity of TNF-α (101,102). Consistent with these reports our result demonstrates that Th2 response inducing helminth infection was associated with enhanced tissue damage while the extent of damage in the lungs of control mice were minimal.

(a)



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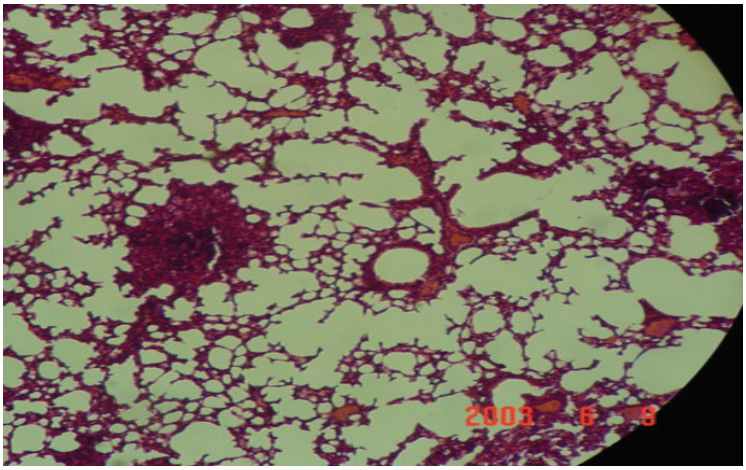
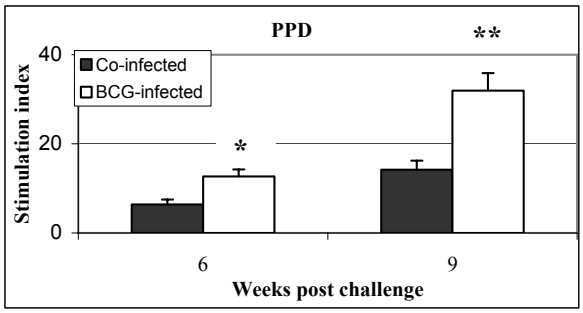


Fig 6 Photomicrographs of lungs of mice infected with *M. bovis* BCG in the presence (A) or absence (B) *of S. mansoni* co infection. The lungs were examined at week 15 post challenge. Five consecutive sections were examined for each mouse. Magnification x100.

6.5 *S. mansoni* impairs proliferative and IFN-g responses of spleen cells to mycobacterial antigen PPD but enhances background Th2 type responses (paper II).

We have earlier proposed that Th2 responses induced by chronic helminth infection could inhibit Th1 responses required for protection against mycobacteria. To investigate whether the poor resistance against BCG infection observed in this study is reflected in the *in vitro* cellular responses to M.tb antigens, we analyzed proliferative and IFN-γ responses of the spleen cells to PPD, the data reveals that there was markedly lower *in vitro* spleen cell proliferative and IFN-γ responses (Fig. 7). Further analysis of Th2 cytokine responses (IL-4 and IL-5) shows that Schistosoma infected animals produce high Th2 cytokine levels while the controls hardly make Th2 responses. This supports the hypothesis that poor resistance against mycobacterial infection is the result of poor Th1 response in helminth infected individuals.

(a)



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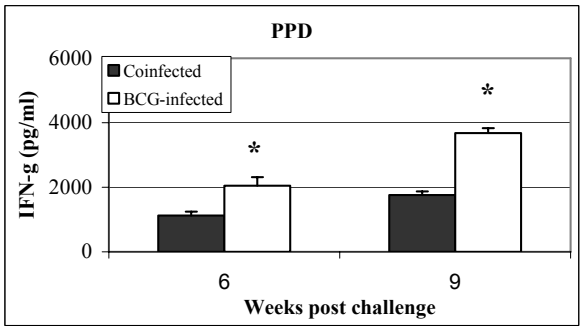

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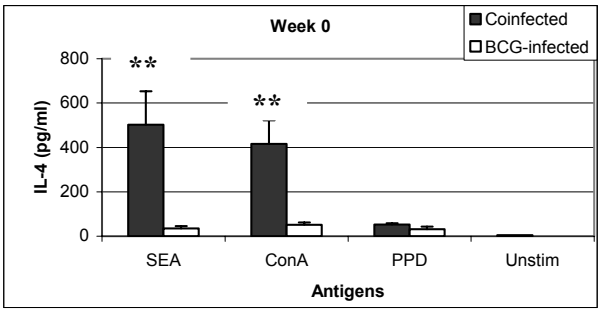


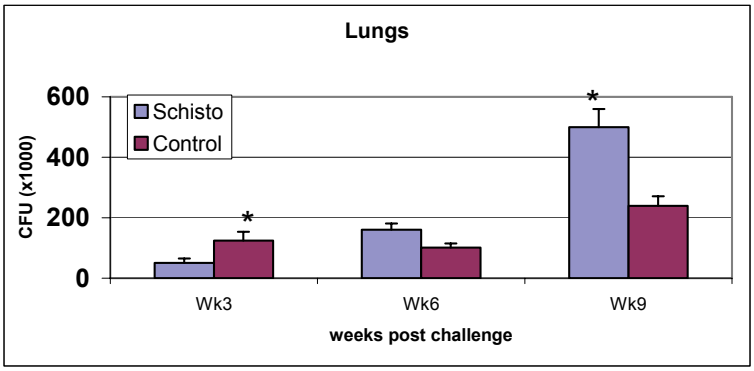
Fig 7 *In vitro* T cell responses in *S. mansoni* infected mice versus controls. (a) Proliferation (b) IFN-γ production (c) IL-4 secretion. Result for proliferation was expressed as stimulation indexes (mean count per minute in stimulated cultures/mean count per minute in un-stimulated cultures) while results for the cytokine levels were expressed in pg/ml. ten animals were used for each experiment. *P<0.05, **P<0.01.

6.6 Chronic *S. manoni* infection reduces BCG induced protection against *M. tuberculosis* challenge (paper III)

In paper I we observed that BCG vaccination in helminth infected individuals failed to significantly improve *in vitro* T cell responses to TB antigens. Whether the poor immunogenicity of BCG in helminth infected individuals is a reflection of poor efficacy is a problem that remains to be addressed. As an initial step to address this question, we investigated whether *S. mansoni* infection in mice could affect the efficacy of BCG against *M. tuberculosis* challenge. We evaluated this by following the kinetics of the growth of TB bacilli, lung damage as well as the *in vitro* Th1/Th2 cytokine responses against TB antigens. The results indicate that *S. mansoni* does indeed reduce the protective efficacy of the vaccine as shown by the high bacterial loads in the lungs and liver (Fig. 8) as well as the extensive lung tissue damage (Fig. 9) caused by *M. tuberculosis* infection in mice vaccinated in the presence of *Schistosoma* infection. The Th2 cytokine IL-4 has been shown to deactivate macrophages, switches of signaling via TLR-2 and potently down regulate iNOS (103) which are crucial for host defense (104). Moreover it has been shown in the mouse model as well as in humans that IL-4 plays a role in tissue damage (105,106).

This is supported by the high bacterial load as well as the greater tissue destruction in mice infected with Th2 inducing *Schistosoma* infection.

(a)



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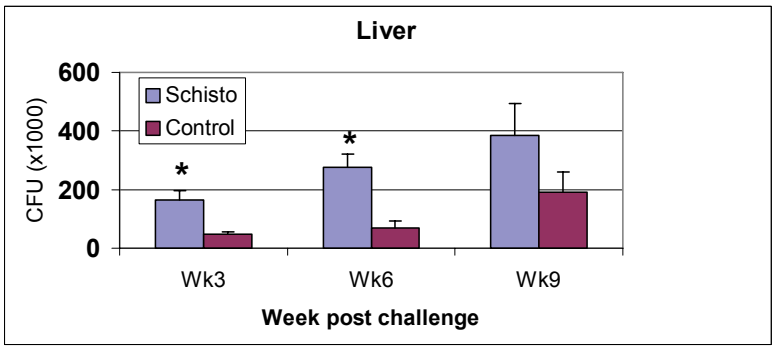
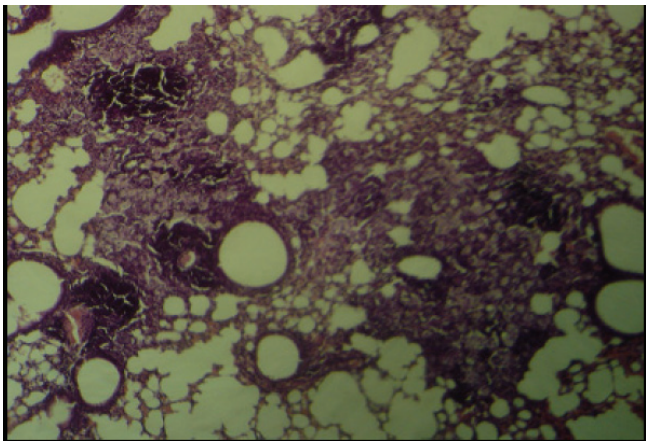


Fig 8 Protective efficacy as assessed by kinetics of the replication of *M. tuberculosis* in the lungs (a) and the liver (b) at weeks 3, 6 and 9 post challenge in mice BCG vaccinated in the presence or absence of *S. mansoni* pre-infection. Results are mean CFU from 5 mice per group± S.E.M.

(a)



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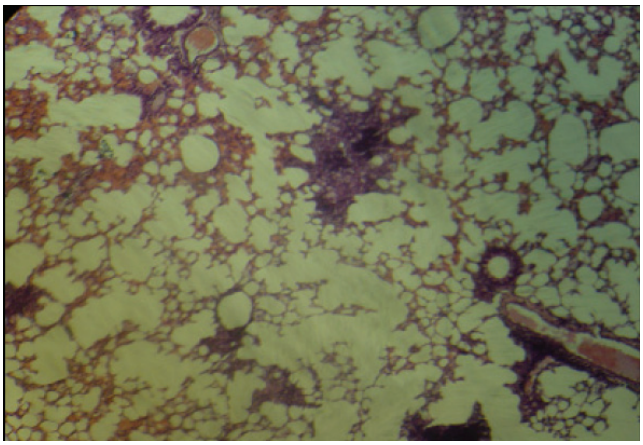
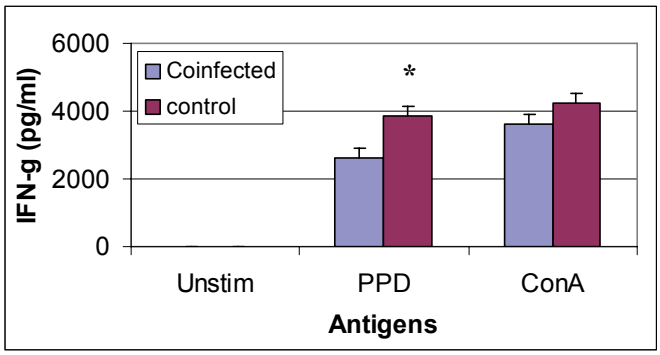


Fig. 9 Photo micrograph of lungs of mice BCG vaccinated in the presence or absence of *S. mansoni* infection. The lungs were examined at week 9 post challenge. Ten consecutive sections were stained and examined in each of the experimental groups. (a) section from mice Schistosoma infected prior to vaccination and (b) section from the control group.

6.7 Chronic *S. mansoni* infection reduces *in vitro* PPD induced IFN-g and nitric oxide production while enhancing Th2 responses (Paper III)

To assess whether the differences between *Schistosoma* infected animals and controls in controlling *M. tuberculosis* infection is reflected in *in vitro* Th1 or Th2 responses, we examined the *in vitro* IFN-γ, nitric oxide, IL-4 as well as IL-5 responses. The result shows that Schistosoma infection does impair Th1 type (Fig 10a & b) responses while at the same time enhance Th2 type immune background. Noteworthy mentioning however is that spleen cells from none of the animals produced significant Th2 cytokine when stimulated with TB antigen PPD while Th2 response to a T cell mitogen con A is several fold higher in *S. mansoni* infected animals.

(a)



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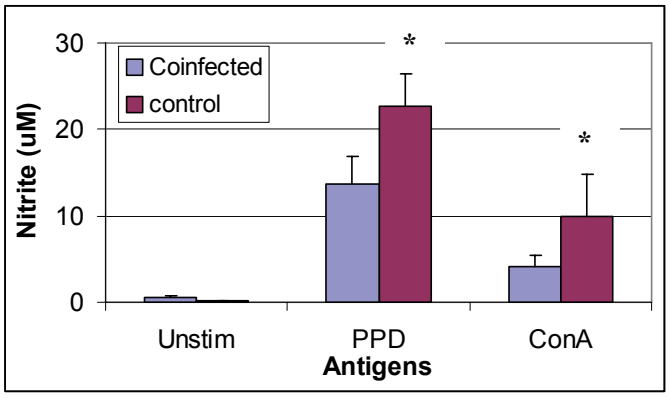

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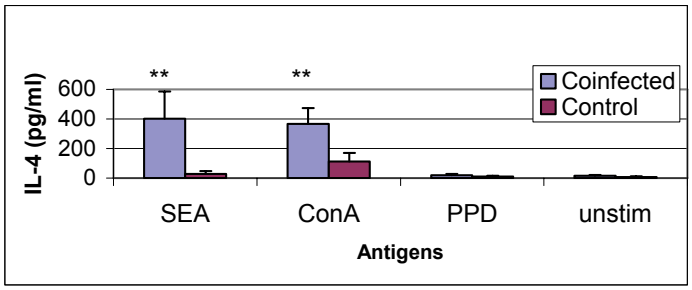


Fig 10 *In vitro* responses of spleen cells as assessed by IFN-γ (a), nitrite (b) as well as IL-4 (c) levels in mice vaccinated with BCG in the presence or *S. mansoni* infection and later challenged with *M. tuberculosis*.

6.8 Helminths are associated with active pulmonary TB independently of HIV infection (Paper IV)

The data reported in paper I show that helminth infection suppresses Th1 type responses to TB antigens. In papers II and III, using *S. mansoni* as a model we have demonstrated that helminths could impair resistance against mycobacterial infections as well as reduce the efficacy of vaccines against the disease. Whether what is observed in *in vitro* systems in humans or in animal models could be relevant in clinical situation remains to be addressed. As an initial effort to address this issue, we conducted a case control study to assess the presence of an association between intestinal helminths and active pulmonary tuberculosis in an area where both pathogens are endemic.

The result shows that indeed the prevalence of worms in active TB patients was significantly higher than their healthy household contacts (70.9% vs. 36.3%, P<0.0001). Conditional logistic regression analysis showed that there is strong association between having active pulmonary tuberculosis and intestinal parasite infection independently of HIV infection status. The odds of being active TB patient increases progressively with increasing number of worm species a person was found to be infected with (Table 1).

A study in South Africa showed the highest incidence of TB in the poorest villages (107). This may appear uninteresting given the notion that TB is a disease of poverty

due to nutrition and overcrowding. However, there was high prevalence of unique strains suggesting reactivation of previous infection as opposed to recent transmission. The communities in this village were highly infested with intestinal helminths supporting our finding that indeed helminths could increase the incidence of clinical TB. This is consistent with the findings of Tristao-Sa *et al* who reported high worm infection rate in TB patients in Brazil (60) and that of Diniz *et al* who observed strong association between intestinal nematode infection and multibacillary leprosy (61).

It should however be kept in mind that since TB is a chronic disease and it is difficult to determine whether or not helminth infection preceded the development of active TB. However, given the observation that helminths modulate immunity by enhancing regulatory T cell activity and as a consequence increase in Th2 type responses, it may be more plausible to think that it is the worm infection which predisposes people to getting active TB.

Assessing worm load may be needed to see if increasing load shows increasing association with active TB to give further support to the hypothesis. Unfortunately we were not able to do that in this study but we have assessed the association between the number of worm species per person and having active TB and the result indicates there is progressive increase in the odds of being active TB patient as the number of worms species increases. This provides further support to the hypothesis that helminth infection may be an important risk factor for the development of active TB.

Table 1 Prevalence of intestinal helminths and HIV1/2 in TB patients and their healthy household contacts.

	Group		OR (95% CI)	
Type of infection	TB patients N (%)	Controls N (%)	Crude	Adjusted
A. lumbricoides	123 (53.5)	101 (19.8)	5.6 (3.7-8.5)	5.7 (3.6-8.9)
Hookworm	65 (28.3)	65 (12.7)	3.0 (1.9-4.9)	3.3 (2.0-5.5)
S. stercoralis	36 (15.6)	20 (3.9)	2.1(1.1-4.1)	2.4 (1.2-5.1)
T. trichuria	10 (4.3)	26 (5.1)	0.7 (0.3-1.7)	0.6 (0.2-1.5)
S. mansoni	9 (3.9)	31 (6.1)	0.3 (0.02-1.3)	0.5 (0.02-1.0)
E. vermicularis	0 (0.0)	6 (1.2)	ND	ND
Number of worm species per subject				
1	103 (44.8)	131(25.7)	3.5 (2.4-5.1)	4.3 (2.8-6.8)
2	47 (20.4)	48 (9.4)	4.9 (2.9-8.4)	4.7 (2.5-8.7)
3^{+}	13 (5.7)	6 (1.2)	11.2 (3.9-32.0)	12.2 (3.9-52.6)
Any worm infection	163 (70.9)	185 (36.3)	4.1 (2.8-5.8)	4.2 (2.7-5.9)
HIV1/2	108 (46.9)	60 (11.8)	7.7 (4.8-12.5)	7.8 (4.8-12.6)

A. lumbricoides =Ascaris lumbricoides, *S. stercoralis=Strongloides stercoralis*, *T. trichiura=Trichuris trichiura*, *S. mansoni=Schistosoma mansoni*, *E. vermicularis=Enterobius vermicularis*
N=number of positive subjects
ND=not done; Adjusted odds ratio were obtained after controlling for age, sex, HIV infection as well as for factors that cluster in house holds

1.1 Poor immunogenicity of BCG vaccination in helminth infested population is accompanied by enhanced in vitro TGF-β production (paper V)

The studies reported in papers I-IV show that intestinal helminths affect immunity against mycobacterial infections or the efficacy of vaccines against them. In an attempt to see how helminths negatively influence the outcome of vaccination against mycobacterial infection, we examined T cell responses following BCG vaccination in worm infected human population and compared the results to treated controls.

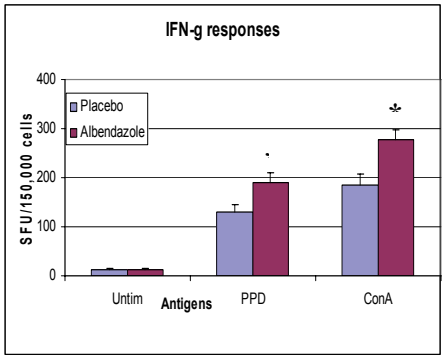
The result confirmed that indeed worms do affect the immunogenicity of BCG vaccination as shown by low TB antigen specific Th1 type responses in worm infected volunteers compared to treated controls **(Fig 11a & b)** consistent with our

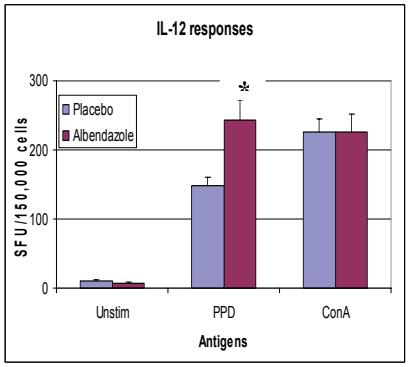
previous reports but the poor response in the wormy population is associated with enhanced production of suppressive cytokine TGF-β **(Fig 11c)**. However, no significant differences were observed with regard to the production of Th2 cytokines (IL-4 or IL-5) between individuals with worm infection compared to treated controls **(Fig 11d & e)**. This may appear surprising given the notion that helminths induce potent Th2 responses and hyporesponsiveness associated with helminth infection is the result of the Th1-Th2 shift of the immune background.

Recent studies in man and primates however show that hyporesponsiveness characteristics of chronic helminth infection is a result of increased Treg activity which non-specifically suppresses both Th1 and Th2 responses (108-110). Tregs induce inhibitory cytokines including TGF-β and IL-10. These cytokines suppress Th1 type responses and interfere with effector T cell activation (109).

In summary, the present study showed that helminth infection were found to induce poor TB antigen specific Th1 responses following BCG vaccination and this was associated with enhanced TGF-β production, a cytokine produced mainly by suppressive T cells. Supporting the notion that chronic helminths impair cell mediated T cell responses to subsequent infections or vaccinations by enhancing suppressor T cell activities in man but not by polarizing the immune balance in favor of Th2 cytokines as frequently shown in the mouse model.

(a) (b)





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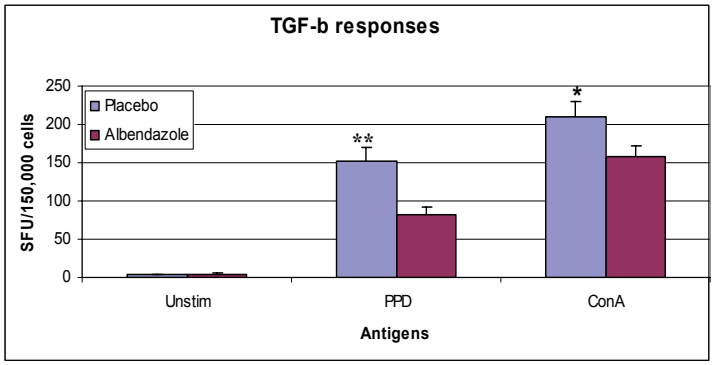
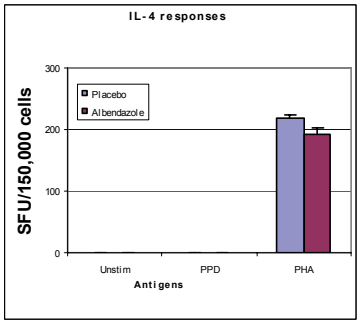


(d) (e)



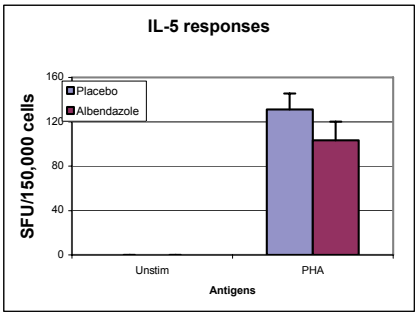


Fig 11 IFN-γ (a), IL-12 (b), TGF-β (c) IL-4 (d) and IL-5 (e) responses in helminth infected individuals vaccinated with BCG. Cytokine levels were assessed using the ELIspot assay and results are expressed as mean number of spot forming units/150,000 cells. *p<0.05, **p<0.01.

7 CONCLUDING REMARKS

The public health importance of TB is huge and helminths are widespread in those same areas where TB is rampant. The impact of concurrent helminth infections on the epidemiology and pathogenesis of TB is an important but neglected area that deserves greater consideration if we are to develop a new and better ways of controlling the disease. At the present time a number of vaccine candidates are entering clinical trials. Indulging into such an expensive venture may prove futile without proper consideration of why the existing vaccine has failed. The widespread existence of helminth infection in areas of high TB incidence and the consequent immunomodulation may affect the ability of the host to respond to subsequent infections including *M. tuberculosis* and available evidence indicates that such infections may impair the efficacy of vaccines against different infectious agents including tuberculosis. This needs to be explored further.

If it is proven that worms are detrimental in affecting susceptibility to or the efficacy of vaccines against diseases of major public health importance, regular mass deworming in addition to its direct health benefit, could also be of relevance in the control of diseases of public health significance.

On the other hand, the interaction between worms and the immune system may have beneficial consequences to the host, as it is a result of a long evolutionary coexistence between humans and worms. It has been suggested that worms provide protection against inflammatory diseases and this has been considered as one of the explanations for the low incidence of atopic and/or autoimmune diseases in the developing world (111). There are some indications for the protective effect of worm infections in reducing Th1 mediated immuno-pathology during inflammatory bowel diseases (79,112,113). Immunization with *S. mansoni ova* was shown to provide protection against experimental autoimmune diseases in mice (76). *Trypanosoma spp* infection was shown to protect against collagen-induced arthritis in rats (114). These observations, although they still need to be confirmed in clinical settings, may suggest that helminths may induce some desirable responses such as suppressing allergic and pathogenic inflammatory responses. This has been raised by some authors as an argument against regular mass deworming (115). However, it remains to be evaluated

whether the public health significance of this could be compared to the substantial negative consequences of allowing people carry worms throughout their lives.

8 FUTURE PERS PECTIVE

The studies outlined in this thesis show that chronic helminth infections affect immune responses against TB as well as the immunogenicity of the vaccine against TB. Further understanding of the mechanism behind this effect would be necessary to avoid future vaccines from falling into the same trap. Our study in humans (Paper V) shows that altered responses following BCG vaccination is associated with enhanced TGF-β levels in helminth infected subjects. This suggests the involvement of regulatory T cells in helminth induced immunomodulation.

The following are some of the questions that need further investigation in connection to this thesis:

- It would be interesting to know if TGF-β is behind the immunosuppression in worm infected population. Could neutralizing TGF-β reverse the situation?
- What roles do regulatory T cells play in helminth induced immunomodulation?
- What is the appropriate time to vaccinate after eradication of helminth infection to obtain optimum protection?
- What are the kinetics of changes of the immune profile following eradication of helminths?
- Does mass deworming change the incidence of TB?
- Is it possible to use adjuvants to reverse helminth induced immunomodulation?
- Can eradication of helminths change the phase of immune disorders including autoimmune and atopic diseases?

9 ACKNOWLEDGEMENTS

Very many people have contributed to get the work to where it is now. I would like to particularly thank:

The volunteers who generously allowed us to use their precious samples for the study.

Sven Britton: I still clearly remember the day I first met you! It has been such a blessing to have you as a mentor!!

Hannah Akuffo: Your continuous supervision and support has been instrumental. Your courage and strength is exemplary. Thank you!

My friends at AHRI: Ebba Abate, Yonas Kassahun, Hiwot Tilahun, Dawit W/M, Liya Wassie, Elena Hailu, Kidist Bobosha, Martha Zewdie, Almaz Kiros, Endalamaw Gadissa, Adane Mihret, Markos Abebe, Abebe Habte, Wude Mihret, Meseret Habtamu, Yusouf Sani, Mesfin Taffese, Edossa Negera, Mekdim Mekdem Mekonnen, Rahel Kebede, Rahel Ewnetu, Girma Berhanu, Wondu Wagaye, Tigist, Alemayehu Kifle, Aklilu Getachew. Such a wonderful atmosphere below the stairs wouldn't have been created and maintained without you.

Sr Genet Amare-never complained about the huge workloads in the field even when we had to deal with hundreds. Thank you!

Isaac Abraham: for the help at the start and for being a good friend ever since

Ameha Kebede: thanks for the sporadic "Gomen Besiga" and for the nice time every time we meet.

Mamuye Hadis: Congrats! Perseverance pays off.

Adugna Woyessa: Good luck with the PhD!

AHRI management: Abraham Aseffa, Howard Engers, Hailu Zewge it would have been difficult without your support and your intellectual contribution has been invaluable.

Eden Emiru (hope your accounting has improved), Lemlem, Haqi T/H, Etetu Gudeta, Emnet Fiseha – thanks for the friendship and support in administration.

Ato Seife was helpful with transportation though Girma had to step-in shortly.

Alemu, Bayleyegn, Ayalew, Muluken, Emawayish, Asayech, Semene, W/O Belaynesh, Saba, W/O Berhane (Adei). Thanks for being so nice to me, I always miss you all.
Bamlaku you have been very pleasant and so cooperative all the time.
Banchiayehu and Kiros thanks for preparing slides in immunopathology studies.

Nunu Worku: I have no words to thank you nunish! Your hospitality and kindness has been extraordinary. I always proudly think I have a sister in Stockholm. Tack!!!
Eleni Aklilu: I have fond memory of my time in Sweden. Thanks for being such a good friend.

Hannah's group: Susanne Nylen, Liv Eidsmo, Gabriel Guzman, Iman Satti very stimulating group to be in. Thanks for helping me settle in and for the scientific discussions.

Getahun Abate and Nani: Thanks so much for your hospitality during my St Louis stay. I really have a very pleasant memory of it.
Daniel Assefa: Thanks Dan for the advice and friendship from Stockholm all the way to St Louis.
Aster Beyene: Congratulations with the baby!!! Just in time!

Gezahegn Gorfu, Wondwossen Sime, Zenebech Wondimu, Zinash, Yemisirach, for being good friends.
Geremew Tasew your long discussions are always entertaining and it is good to have you around during the write-up.

Dan Hoft (SLU, St Louis): Thanks for being so generous with your time, reagents, knowledge and for the nice lunch on my departure.
Chris Eichoff, Azra Blazevic, Olivia Giddings for helping me learn a lot in the short time I was with you in St Louis. What a nice atmosphere to work with you!

Thomas Schoen: Thanks for the productive collaboration from Gondar, Linkoping and Kalmar and for being a very dependable friend. I enjoyed the discussions we always had and thanks for introducing me to Maria, Freja (such a bright kid!) and Algot.

Helena Hellqvist: Thanks for the friendship and for introducing me to Sweden, the dinners and visits to the archipelagos

Andrzej Pawlowski: for the help with organizing the huge workload in the TB lab during the mouse experiments and for the nice chat every time we met.
Cecilia Thors: for teaching me how to get schisto out of the snails.
Melles Haile: for the productive collaboration and for the help with the mouse work.
Gunilla Kallenius: for allowing me to work in the TB lab.

Mats Wahlgren Group: Mats Wahlgren for being so welcoming and nice! Qijun Chen, Victor Fernandes, Andreas Heddini, Sanjay Ahuja, Marianne Lebadd, Niloofar Rasti, Fredrik Petersson, Malin Haeggstrom (for the chat once in a while), Silvia Botero-Kleiven, Nocolas Joannin, Fingani Mphunde, Kristen Moll, Daniel Palm, Staffan Svard, Anna Vogt, Malin Weiland, Mobo Mok. Tack!
Inger Becker (for quick help with my requests), Johan Lindh (for reminding me some Amharic words once in a while) Inger Ljustrom, Lisbeth Gregory.

Ginci Health Center staffs: **Addisu Girma, Shibiru Kumsa, Abubeker Kedir, Negussie, Amsalu, Yordanos, Tadesse**- I value your contributions a lot.
Dawit Wolday: Sorry for the unsporting behavior at Meskel square.

Anna Logdberg, Eva Pijnenburg, Kjerstin Bjorkholm, Annita Wallentin for all the help in the administrative issues which I had no clue about.
Ulf Bronner for being friendly and help when I had the headache and for the recommendations

My father: who kept telling me that I'm capable of bigger things. I miss you big!

Duretti: your coming to this world has changed my perspective of life.

Hawi: I need to thank you the most as the world turned a much better place after you came in.

This work was funded by grants from the Swedish International Development Agency (Sida), Sequella Global Tuberculosis Foundation, USA, World Health Organization/Tropical Disease Research (WHO/TDR), Armauer Hansen Research Institute (AHRI), the Swedish Institute, Research and Publication Office, Gondar University and Karolinska Institute.

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