The Impact of Helminths on HIV, Measles, and Tetanus-specific IgG Antibody Responses among HIV Co-infected Adults in Kenya

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2013

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Program Authorized to Offer Degree:

Public Health – Epidemiology

University of Washington

Abstract

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In Africa, helminth and HIV infection is highly prevalent, making co-infection with helminths and HIV likely common. Both infections impact the host immune response through immune suppression and dysregulation, which also may have implications for vaccine efficacy and efforts to decrease vaccine preventable deaths. We sought to determine the impact of helminth infection on humoral immune responses to HIV and to previously administered measles and tetanus vaccines. We developed a method to evaluate HIV-specific antibody responses using protein microarray and principal components analysis, and identified distinct patterns of HIV-specific antibody responses that correlate with concurrent viral load and subsequent change in CD4 count. Within two

randomized trials examining the effect of deworming on HIV disease progression, we conducted nested serologic studies to examine the impact of helminth infection (n=100) and deworming (n=35) on specific antibody responses. The prevalence of HIV specific antibody responses was similar between individuals with any helminth infection compared to helminth uninfected individuals. However, those with schistosomiasis infection had significantly lower HIV specific antibody responses compared to helminth uninfected adults. The prevalence of measles and tetanus antibody responses were similar between helminth infected and uninfected individuals. Comparing dewormed to placebo treated Ascaris and HIV co-infected adults, changes in measles and tetanus antibody responses over 3 months were comparable between treatment groups. Though helminths did not alter antibody responses to previously administered measles and tetanus vaccines, antibody responses to concurrent HIV infection may be diminished in individuals who are co-infected with some helminth species.

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INTRODUCTION

Often described as ancient diseases of poverty, the soil-transmitted helminthiasis (STH) and schistosomiasis refer to a group of infections within the larger classification of neglected tropical diseases. The impact of these diseases is far reaching, with estimates of over 1 billion people affected globally, mostly in Africa and South-East Asia [1]. The World Health Organization set a goal in 2001 to provide 75% of school-aged children at risk of infection access to treatment by 2010. Falling short of the goal, in 2009 it was estimated that of those who required preventive chemotherapy for soil-transmitted helminths and schistosomiasis, only 31% and 8% received it, respectively [1].

The importance of regular treatment with antihelminthic medication is well known, however, our understanding of the impact of helminths on the host is still evolving. Helminths produce chronic infections by modulating the host's immune response [2], which may have profound effects on other common diseases in endemic areas, including HIV [3,4,5], as well as immunizations [6,7,8,9,10,11]. In a systematic review assessing randomized control trials that evaluated the impact of deworming on markers of HIV progression, a benefit on viral load and CD4 count was seen in 3 trials, which pooled together was a significant result [4]. Additionally, immunizations are of particular importance for HIV infected individuals due to HIV-induced immune dysfunction, which results in an increased vulnerability to many diseases, including many which are preventable by available vaccines [12,13,14]. If helminths induce immune responses that worsen HIV disease and decrease vaccine responses then eliminating helminth infections in HIV co-infected individuals may potentially be a cost-effective intervention to improve host control of HIV infection, as well as vaccine efficacy.

This study aims to examine whether helminth infections impact host humoral immunity to concurrent HIV infection and previously administered measles and tetanus vaccines. The interpretation of complex immune responses, such as HIV antibody responses in relation to HIV disease progression, requires novel methods for analysis. To assess HIV-specific antibody responses, methods using protein microarray and principal components analysis were developed to identify clusters of HIV antibody responses that were associated with disease progression.

To determine whether helminths alter HIV or vaccine-specific antibody responses among HIV infected individuals, we conducted two nested serologic studies. Helminth infected and uninfected individuals were identified from the control arm of a randomized clinical trial (RCT) evaluating the impact of empiric deworming on HIV disease progression. The prevalence of HIV and vaccine specific antibody responses was compared by helminth infection status, including species-specific infection. In a second RCT, changes in HIV and vaccine specific antibody responses were evaluated among dewormed or placebo treated adults with HIV and Ascaris (a type of STH) co-infection adults after 3 months of follow-up.

BACKGROUND

HELMINTH INFECTION

The four most common species of soil transmitted, parasitic worms are *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms, which include *Necator americanus* and *Ancylostoma duodenale*. Infections by these helminths affect roughly a quarter of the world's population, many of whom reside in developing countries [15] (Figure 1). Two major determinants of helminth transmission are climate and the environment. Helminths thrive in tropical and subtropical regions with moist soil and warm temperature, specifically in poor communities with little clean water and poor sanitation. Helminth infections cause more morbidity than mortality, with the degree of morbidity related to the intensity of infection. Peak intensity of infection varies by species. Ascaris and Trichuris infect mostly children 5-15 years with decreasing infection intensities in adulthood. Hookworms can infect heavily in children but also remain high in adults [15]. Annual deaths due to helminths have been estimates between 12,000 and 135,000, while the estimated DALY lost to helminth infections are between 4.7 and 39.0 due to varying estimates of the severity of infection [15]. Health effects of helminth infection include anemia and protein loss, which can result in malnutrition and delayed cognitive development in heavily infected children. Although treatment for helminth infection can reduce transmission intensity among populations, reinfection following deworming can occur rapidly [15]. As a result WHO recommends annual deworming of school aged children, eliminating the health effects of severe infection [16].

HIV AND HELMINTH CO-INFECTION

Considerable geographic overlap exists among HIV and helminth infected individuals. Of the 22 million Africans estimated to be infected with HIV [17], many are likely co-infected with at least one species of helminth [18].

Helminth infection may have adverse effects in people also infected with HIV. Co-infected pregnant Kenyans in an observational study had greater odds of mother-to-child transmission of HIV than helminth free women with HIV [3]. In addition, some studies suggest that helminths are associated with increased HIV disease progression in HIV infected individuals, leading to lower CD4 counts and higher viral loads [4]. Albendazole treatment of HIV infected individuals results in significant reductions of IL-10, compared with individuals receiving placebo ($p=0.04$) [19]. Deworming HIV-infected individuals has also been shown to decrease HIV-1 viral load and may impact other markers of HIV disease progression, such as CD4 count [4].

Both HIV and helminths possess mechanisms to control the complex interplay between the host's ability to clear a chronic infection and its need to minimize damage due to continued inflammatory responses (Table 1). HIV, through the adaptive immune response, continually switches between immune activation and immune suppression. Helminths on the other hand, have developed an array of immune-modulatory proteins that drive the innate immune response into an immunosuppressive state. Epidemiologic studies suggest an increased progression of HIV disease in the presence of helminth infections, although how these mechanisms compete in dually infected individuals is not fully understood immunologically.

Host Immune Responses to HIV

Typically, HIV infection results in high viral replication, a decline in CD4+ T cells, leading eventually to AIDS [20]. While both cellular and humoral immunity are involved in the control of HIV infection,

the role of the humoral response in controlling HIV is less well understood. Neutralizing antibodies are integral to blocking new infection while cytotoxic T lymphocytes are more involved in control of already established infections [21]. High levels of neutralizing antibodies do not appear to be protective of HIV-1 disease progression [21]. Multiple studies looking at broadly neutralizing antibodies have observed lower titers of neutralizing antibodies among elite suppressors compared to progressors [20]. This may be because suppressors control the infection at onset, never allowing the humoral response to generate a broad antibody repertoire [22].

Dysregulation in global B cell function has been examined in HIV infection [23]. Hypergammaglobulinemia, or elevated IgG levels, have been reported in most HIV infected individuals, including among long-term nonprogressors, beginning in primary HIV infection [24]. HIV infection induces polyclonal activation of B-cells in the blood and gut, including those specific for third party and auto-antigens [25,26]. Cross-reactive neutralizing activity was significantly associated with lower CD4+ T cell counts suggesting disease progression [21]. Moreover, enhancing antibodies, as opposed to neutralizing antibodies, may bind to virus less avidly bringing the virus into cells. These antibodies are present in individuals as they advance in disease as this antibody-mediated enhancement may be adding to progression [27].

HIV infection is characterized by immediate high viral replication followed by host immune activation to control the infection and limit viral activities. With chronic infection, immune suppressive controls such as T regulatory cells increase to minimize the damage of inflammatory responses, with the consequences of also leading to decreased anti-viral immune responses and increased disease progression [28]. Disease progression is marked by CD4+ T cell depletion, CD8+ T cell expansion and chronic immune activation [29].

T regulatory cells (T-regs) and Th17 cells are a subset of CD4+ T cells with important roles in immune control and have recently received more research attention [30]. These two cell types appear to have opposing purposes with T-regs involved in immune suppression and Th17 cells involved in mounting strong inflammatory responses. However, Th17 and T-reg cells share a common developmental link [31,32]. Immunity at mucosal surfaces is compromised by the absence of Th17 cells. Among HIV infected individuals, Th17 cells are depleted with advanced disease, but are preserved in slow progressors and elite controllers [30]. A study looking at the number of Th17 cells present in acute infection found low numbers of Th17 cells to be predictive of T-cell activation in the following year, another marker of disease progression [33]. IL-17 also has a structural role in the maintenance of tight junctions between intestinal epithelial cells, as inhibition of IL-17 signaling worsens epithelial destruction in colonic injury disease models [30].

Host Immune Responses to Helminths

Helminths are extracellular eukaryotic parasites with large genomes that vary greatly in their biology but evoke similar host immune responses. Among some, parasitism leads to complications caused by the blockage of internal organs or the effects of pressure exerted by growing parasites. Parasite eggs migrating through body tissues also cause direct tissue damage, as well as initiate hypersensitivity reactions leading to indirect tissue damage [2]. Most infected individuals however, are asymptomatic and act as long-term reservoirs for transmission [34].

Immune modulation due to helminth infection results in general immune hyporesponsiveness and anergy of the host [2]. Helminth induced immune modulation is represented by high levels of pro-inflammatory and immunosuppressive Th2 cytokines [35,36,37], creating an ideal environment for chronic infection. The polarized Th2 response down-regulates Th1 responses along with associated

cell and cytokine subsets [37]. The characteristic immunosuppressive response caused by helminths involves Th2 and T-reg cell types, which is related to the intensity of infection. In some animal models, the infective stage of the parasite stimulates a Th1-like response, and it is only after the infection is established and adult worms are present that the Th2 switch predominates [34]. However, this observation is not observed in all animals. Among the many actions employed by helminths to suppress the immune response are: an increased threshold for effective immune activation of T cells, defective intracellular signaling, decreased numbers of costimulatory molecules, increased numbers of T-regs, increased intracellular negative regulation of T cell activation in T cells, dysregulation of cytokine secretion, and a T cell imbalance [2].

An important action by helminths is the use of helminth proteins and products to interact with host innate immune responses (Table 2). Their interaction can occur through dendritic cells, toll-like receptors and costimulatory receptors to trigger Th2 and T regulatory responses. Dendritic cells (DC), through their signaling and antigen presenting, are largely responsible for the polarization of T cell development towards Th1, Th2, or T-regs. Helminths and helminth products modulate DC function, such as DC maturation, as well as are involved in signaling through TLRs, such as TLR-2,3, and 4. TLRs mostly associated with signaling Th1 responses are activated by helminth products to signal Th2 and regulatory responses [38]. Additionally, helminth products are able to avoid the normal TLR-induced MAP kinase and NF-kB signaling pathways resulting in immature antigen presenting cells and decreased pro-inflammatory cytokines [39]. The use of helminth products to stimulate anti-inflammatory immune responses is being investigated to treat auto-immune diseases and severe allergies [39].

Impact of Helminths on HIV

Dual infection with HIV and helminths may have adverse impacts on HIV susceptibility, progression, and transmission. The acquisition of HIV may be increased by the presence of a helminth infection and its associated Th2-like immune environment. A study looking at cells from persons with schistosomiasis, intestinal helminths, or filariasis found that these cells were more susceptible to HIV-1 infection *in vitro* than cells from persons without helminth infection [37]. It is possible that helminths increase the level of chemokine receptors that serve as HIV-1 co-receptors through the Th2 immune responses and signaling. Demonstrating this association, an increased density of CXCR4 and CCR5 on the surfaces of cells was seen from patients with helminth infection and may be the result of up-regulation by the Th2-associated cytokines IL-4 and IL-10 [37]. If correct, a more rapid increase in HIV prevalence in areas with high helminth burdens may be due to pre-existing helminth infections.

HIV progression is slowed or deterred by Th1 and CTL responses. Th1 cellular responses are necessary to control helminth burden and HIV viral replication [37,40]. The shift to a Th2 immune environment due to helminths may lead to faster HIV disease progression, however, the current evidence is not conclusive. Several studies have found treatment of helminths to be associated with decreased HIV progression measured by viral load and CD4+ count [41,42,43,44], while another study found no association [45], and others found an increase in disease progression [46,47]. All three of the randomized controlled trials support a beneficial effect of deworming on HIV disease [4]. Additionally, deworming HIV infected individuals with albendazole resulted in a significant reduction of IL-10, compared with individuals receiving placebo [19]. Two more studies claimed to find an increase in viral load with deworming; however no comparison groups were evaluated [48,49]. Interestingly, an experimental model in primates showed co-infection with SHIV and *S. mansoni* increased the expression of Th2-associated cytokine responses and viral load in acute

SHIV infection compared to only SHIV-infected animals. Additionally, chronically SHIV infected animals had an increase in viral load when challenged with *S. mansoni* infection [50].

If helminth infections cause increased viral loads, an increase in viral shedding and transmissibility of HIV could result [37]. However, an alternative theory is that treatment of helminths may release parasite antigens, increasing Th2 activation and HIV replication. Additionally, helminth elimination may result in a decrease in immunosuppressive cytokines like IL-10 and TGF-b, which may also increase immune activation and viral replication [37]. More research on the consequences of dual infection is needed.

VACCINE RESPONSES AND CONCURRENT INFECTIONS

Understanding correlates of protective immunity following vaccination is an area of extensive research [51]. For most vaccines currently used, preventing infection relates to the production of vaccine-specific antibodies at the site of replication [52]. However, little was known about protective immunity when the first vaccination was developed; successful smallpox vaccination was determined by the development of a vesicular lesion at the site of scarification [53]. Even today, clear markers of protective immunity are not known for most available vaccines. For tetanus and measles vaccines, the protective immunity level of 0.01 IU/mL and 200 mIU/mL was determined from animal studies and observational studies in humans, respectively [53]. Correlates of protection for BCG are still largely unknown, although this is one example where cellular immunity is thought to play a larger role [52].

Vaccine efficacy is an extension of protective immunity. Vaccines need to prevent infection many years after vaccination. Memory B cells and plasma cells are important in this process. Memory B cells do not actively secrete antibodies until triggered to do so, while plasma cells continually

secrete antibody without further stimulation [54]. Even without circulating antibodies, the presence of vaccine-specific memory B cells can provide protective immunity following infection [55]. Antibodies against many childhood vaccines can be detected in plasma from adults, who receive vaccinations in early childhood [54,56]. The half-life of the measles antibody is thought to be around 200 years while that of tetanus is around 11 years, demonstrating the difference in vaccination mechanisms [56].

HIV Infection Decreases Immune Responses to Vaccines

Diminished immune function among HIV infected individuals even after antiretroviral therapy (ART) initiation increases vulnerability to vaccine preventable diseases [12,14]. HIV infection causes persistent systemic immune activation with a continuous decrease in CD4 T cells and progressive immune dysregulation [2]. As a result of this immune destruction, children with HIV are more susceptible to many infections, some of which are vaccine preventable. For example, HIV-infected children have a 42-fold greater risk of developing invasive pneumococcal disease and an over 10-fold greater risk of developing pneumonia than HIV-uninfected children [57,58]. They also have high rates of mortality when infected, with the odds of death from measles more than doubled compared to uninfected children [59]. Even with ART induced immune reconstitution, an HIV infected child will not reach an equal level of immune responsiveness as an uninfected child, increasing the likelihood of death from childhood illness [12,14].

The weakened immunity that renders children with HIV more vulnerable to infection also decreases their responses to vaccination [14,60]. Effective immunization requires strong vaccine recognition by the host, followed by long lasting cellular and humoral immune mechanisms. HIV infected children display weakly binding vaccine-specific antibodies that wane quickly [60]. A lack

of robust or long lasting immune function inhibits a protective immune response, further increasing susceptibility to vaccine preventable illnesses. As a result, among fully immunized children, those with HIV are at greater risk of vaccine preventable disease compared to those without HIV.

Helminths May Also Diminish Vaccine Efficacy

Helminth infection may compromise host responses to vaccine antigens. Ecologically, regional variation in vaccine efficacy coinciding with variation in the prevalence of enteric pathogens, is observed with many vaccines [61]. A variation as high as 50% in the performance of the rotavirus vaccine has been seen, with highest efficacy in developed countries and lowest efficacy in Africa and Asia [62]. Polio eradication continues to be challenged by the highly diminished and variable efficacy of the oral polio vaccine in India, compared to the rest of the world [63]. A meta-analysis evaluating BCG vaccine effectiveness reported that geographic latitude explains 41% of the variance in vaccine effectiveness, with effectiveness positively correlated with increasing distance from the equator [64]. The distribution of soil-transmitted helminths suggests that helminth infection may be an important determinant of a child's response to vaccination [65].

Studies have demonstrated that deworming before vaccination is associated with higher post-vaccination antibody levels, compared to no deworming [6,7,9,11,66] or deworming after vaccination [66]. Experimental studies in humans and animals have demonstrated that deworming before immunization increases protective antibody titers and interferon-γ levels, while decreasing immunoregulatory IL-4 and IL-10 levels [6,7,8,9,10,11]. While human data examining vaccine efficacy and helminth infection are limited, children with ascariasis who received albendazole prior to receiving oral cholera vaccine were more likely develop a detectable antibody response (seroconvert) than children who were not dewormed [9]. Additionally, children who were

unresponsive to vaccination with trivalent oral poliovirus were more likely to harbor intestinal parasite infections than vaccine responders [67].

Combination of Protein Microarray and Principal Components Analysis

Protein microarray is a technique that can examine many antibody responses simultaneously. The large amounts of data generated from microarray analyses can create data problems with multiple comparisons and multicollinearity. Combining data reduction analyses with protein microarray allows the thorough investigation of antibody responses to HIV disease but in a concise and simple analysis.

Strengths and Limitations of Microarray Studies

Microarray technology was one of the first high-throughput methods for examining differences in biological conditions, tissues, disease states, and individuals [68]. One of the earliest uses of a protein microarray was to examine antibody specificities to an array of denatured recombinant proteins [69]. Unlike genome and DNA sequencing studies, the data generated from microarray studies is only meaningful in the context of how the samples are obtained, as comparisons in physiologic conditions are the goal. Genome data is the same for an organism regardless of its biological state, while the physiologic conditions are short lived and constantly changing [68]. Beginning with how the microarray was built, continuing to how the samples were collected and prepared, and ending with how the data was analyzed, every step may vary from experiment to experiment and can affect the results of the study.

The development of a protein microarray begins with what is attached to a 2-dimensional solid or 3-dimensional liquid phase matrix for screening. Proteins are produced *in vivo* using yeast, *E. coli* or

plant systems, or using *in vitro* transcription/translation systems [70]. Factors that differ by system include protein length, localization, post-translational modification, and protein folding [71,72]. Once the proteins are made, they are affixed to slides and printing can vary from batch to batch. Not detecting a signal at a specific protein spot can mean any one of the following: the protein was not printed, the protein was not folded in the proper way to be recognized, or the antibody being screened for is not present.

Many important issues can vary the final results from a microarray once the raw data are created. The raw signal intensities need to be normalized in a meaningful way in order to separate background noise from true signal. The quantification of the signal does not translate to an objective unit of measurement, but rather relative changes, and may be challenging to interpret and compare across studies. Statistical considerations related to the numerous endpoints measured include addressing multiple comparisons and what analysis plan is appropriate to identify, quantify, and compare patterns of detected antibodies [73].

Methods to Decrease the Dimensionality of Data

Techniques to reduce the dimensionality of data are common and used in many settings [74,75,76]. Data reduction algorithms can become exceedingly complex with applications in text mining, facial recognition, machine learning, and intrusion detection [77]. Reducing the dimensionality of data allows fewer comparisons to be made in regression modeling, which can alleviate issues of multiple comparisons. Additionally, when data on many variables are collected, variables may capture similar information, causing redundancy in what they measure and model issues with multicollinearity [78,79].

Principal Components Analysis (PCA) is a descriptive method of reducing the variation among a set of original variables into components with no assumptions of underlying structures [79]. The goal is to collapse a large set of variables into a smaller number of uncorrelated factors, which represent a large proportion of the variability of the dataset. The factors, or new variables, are linear combinations of the original variables. If a dataset contains n observations on p variables, then n points can be plotted in a p-dimensional space. The first factor is the longest axis of the ellipse that captures as much of the variability in the dataset as possible. The second factor is the second longest axis of the ellipse that is perpendicular to the first axis, ensuring the new factors are uncorrelated and represent distinct aspects of the underlying data [75]. PCA describes the p axes of the ellipse formed by the new factors. The relative lengths of the axes are called the eigenvalues, and a "loading" is the correlation between a factor and the original variable. The original variables are grouped into factors so that the loadings are either high or low in absolute value, which then forms a single score to describe the grouping of original variables [75]. PCA scores can then be used in regression analyses, though the interpretation is challenging. A benefit of PCA is that no probabilistic assumptions need to be made on the distribution of variables or the underlying structures. However, a limitation is that it does not take into account the responses or outcomes in the reduction technique [79]. Using both exploratory analyses along with knowledge of biological function to interpret the groupings, may inform one of unknown underlying patterns among large datasets.

This project is ideal for data reduction techniques such as PCA [78]. There are a large number of variables of interest due to the use of protein microarrays and, though not more than the number of samples examined, still too large for traditional univariate statistical techniques without considerations for multiple comparison. Further research into HIV humoral immune responses in

relation to HIV disease progression is important and exploratory analyses looking for unknown patterns in antibody expression may be informative. Additionally, there are known relationships among the variables of interest, related to HIV virus clade, gene, and protein function. This *a priori* knowledge may inform more predictive components or factors, and may allow the results to be more interpretable. Techniques to reduce the dimensionality of the data will be used to identify underlying structures in humoral immune responses to HIV that are related to HIV disease progression. Using the identified patterns of progressive HIV antibody responses, associations with helminth infection and deworming will be examined.

CHAPTER 1: USE OF PRINCIPAL COMPONENTS ANALYSIS AND PROTEIN MICROARRAY TO EXPLORE THE ASSOCIATION OF HIV-1-SPECIFIC IGG RESPONSES WITH DISEASE PROGRESSION

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Abstract:

The role of HIV-1-specific antibody responses in HIV disease progression is complex and would benefit from analysis techniques that examine clusterings of responses. Protein microarray platforms facilitate the simultaneous evaluation of numerous protein-specific antibody responses,

though excessive data is cumbersome in analyses. Principal components analysis (PCA) reduces data dimensionality by generating fewer composite variables that maximally account for variance in a dataset. To identify clusters of antibody responses involved in disease control, we investigated the association of HIV-1-specific antibody responses by protein microarray, and assessed their association with disease progression using PCA in a nested cohort design. Associations observed among collections of antibody responses paralleled protein-specific responses. At baseline, greater antibody responses to the transmembrane glycoprotein (TM) and reverse transcriptase (RT) were associated with higher viral loads, while responses to the surface glycoprotein (SU), capsid (CA), matrix (MA), and integrase (IN) proteins were associated with lower viral loads. Over 12 months greater antibody responses were associated with smaller decreases in CD4 count (CA, MA, IN), and reduced likelihood of disease progression (CA, IN). PCA and protein microarray analyses highlighted a collection of HIV-specific antibody responses that together were associated with reduced disease progression, and may not have been identified by examining individual antibody responses. This technique may be useful to explore multifaceted host-disease interactions, such as HIV co-infections.

Introduction:

HIV-1-specific antibodies may be important for long-term control of HIV-1 progression, as well as contribute to protection from transmission [80,81,82]. During the course of HIV-1 infection, diverse combinations of antibody responses to specific HIV-1 antigens are produced, with variable intensity and duration [83]. For example, anti-Env IgG is produced and maintained throughout disease, while anti-Gag IgG appears to decrease as HIV-1 disease progresses, independent of changes in HIV-1 plasma RNA [84]. Although antibody responses to select HIV-1 antigens have been investigated in relation to disease progression, sample numbers and definitions of disease progression vary [84,85]. In addition, there has been limited opportunity to investigate the role of combinations of antibody responses on HIV-1 disease progression.

Profiling HIV-1-specific binding antibodies using protein microarray technology may give more comprehensive insight into the role of humoral immune profiles on disease progression. Microarray analyses generate many variables of interest, which may be handled by correcting for multiple comparisons when the goal is inference at the variable level. Consideration of all variables as a whole; however, may be more illustrative of what is occurring in the host. Principal components analysis (PCA) is a useful tool to reduce multivariate responses into fewer composite variables that account for most of the variance in a dataset and has been used in immunosignature studies [86]. Exploring HIV-1-specific humoral immune profiles with PCA and protein microarrays may be a useful way to examine changing immune responses in complex systems, such as chronic HIV-1 infection.

Within a nested cohort study, we assessed the feasibility of utilizing protein microarray and PCA to explore HIV-1-specific antibody responses during disease progression. Using PCA to identify

patterns in humoral responses, we investigated the association between HIV-1-specific antibody responses and markers of HIV-1 disease progression (namely CD4 count and plasma HIV-1 RNA).

Materials and Methods:

Study design

A nested cross-sectional analysis was conducted on 100 stored samples from a large randomized controlled trial evaluating the effect of empiric deworming on markers of HIV-1 disease progression in Kenya [87]. Plasma samples were collected between February 2009 and July 2010. All individuals provided written informed consent to participate in the study. The trial was independently approved by the IRB at University of Washington and the Ethical Review Board of the Kenya Medical Research Institute. The parent trial was registered as NCT00507221 at http://clinicaltrials.gov. The parent study is now complete and significant differences between deworming treatment arms were not found for any HIV endpoints examined [87].

Population

Study participants were enrolled from three sites in Kenya (Kisii Provincial Hospital, Kisumu District Hospital, and Kilifi District Hospital) who were HIV-1 infected, older than 18, were not pregnant, did not meet criteria for ART initiation based on Kenyan Ministry of Health guidelines, had not used ART in the past, and were willing and able to give informed consent. From this population, participants were excluded who had started ART prior to their 12 month visit, did not have a 12 month visit by July 2010, were not from the Kisii or Kisumu study sites, had an abnormal clinical finding at the month 12 visit, took deworming medicine outside of the study, or stopped taking the study medicine before the 12 month visit. From the remaining 329 eligible participants, 100 patients were randomly selected using computer generated random sampling; 25 participants

from each study site and treatment arm. Of the 100 samples selected, 7 were unreadable by microarray and 1 participant was later found to have started ART before the 12 month visit so was not included in the analyses.

Data collection

HIV-1 specific antibody responses were measured from samples collected at the 12 month study visit. Primary endpoints of the parent study were time to CD4 count <350 cells/mm^3 and a composite endpoint of first occurrence of: 1) CD4 count <350 cells/mm^3, 2) first reported use of ART, or 3) non-traumatic death. No deaths occurred among the participants evaluated. Demographic measures were collected at enrollment, while changes in health were evaluated at follow-up visits. CD4 count was measured at months 0, 6, 12, 18, and 24, and viral load (log_{10} plasma RNA) was measured at months 0, 12, and 24 to monitor HIV-1 disease. In this sub-analysis, we consider 12 months as baseline and 24 months as endpoint.

Microarray construction

The HIV-1 microarray was constructed by amplifying and expressing the open reading frames encoding 16 HIV-1 proteins and 13 gene fragments from each of the five major subtypes (A1, A2, B, C, D), or clades of HIV-1. Subsequently, the expressed proteins were printed onto nitrocellulose-coated slides. A total of 143 HIV-1 proteins or protein fragments from 5 virus clades and all 9 viral genes were printed on the arrays.

Full-length infectious molecular clones of HIV-1 were used as templates for polymerase chain reaction (PCR) to create each protein or peptide-encoding gene or gene fragment. The templates used were HIV-1 clones 92UG037, subtype A1; 94CY017, subtype A2; JR-CSF, subtype B; MJ4, subtype C and 94UG114, subtype D. All templates were obtained from the AIDS Research and

Reference Reagent Program except JR-CSF (from Dr. Irvin Chen, UCLA). Primers were designed to contain twenty base pairs of homology to each ORF being amplified and thirty-three base pairs of homology to the plasmid vector, pXT7. The standard PCR cycle was 95°C for 5 minutes followed by 39 cycles of 95°C for 20 seconds, 50°C for 30 seconds and 72°C for 30 seconds for every 500 base pairs of product desired, followed by a final extension of 72°C for 10 minutes. Amplification success was determined by PCR product visualization following agarose gel electrophoresis.

PCR products were inserted into the expression vector, by *in vivo* recombination as previously described [88]. Genes and gene fragments encoding HIV-1 proteins and protein fragments in pXT7 were expressed in an *E.coli*-based cell-free coupled *in vitro* transcription-translation (IVTT; Roche) reaction, solubilized with Tween-20 and printed on nitrocellulose-coated FAST slides (GE-Schleicher and Schuell) using an Omni Grid 100 microarray printer. Microarrays were probed using human sera or control antibodies as previously described [88]. Microarrays were scanned using a ScanArray 4000 machine. QuantArray software was used to quantify the intensity of the spots on the chip. The signal intensity of the "No DNA" controls were averaged and used to subtract background reactivity from the unmanipulated raw data. One third of the spotted proteins had signals greater than the average of "no DNA" control reactions plus 2.5 times the standard deviation, and were considered sero-reactive. HIV uninfected controls were evaluated using the same batch of microarray slides to demonstrate specificity in antibody reactivity (Figure 2).

Data and statistical analysis

Principal component analysis (PCA) was used to reduce dimensionality of the HIV-1 specific antibody responses. All 40 sero-reactive antigens were used to generate the total antigen principal components, which base grouping on the variance of each antigen rather than a pre-specification. Components were retained if at least 3 variables loaded, and the eigenvalue was greater than 2 or

the proportion of variance explained was >5%. Utilizing the hierarchical structure within the HIV-1 antigens, gene-specific PCA was also performed. All sero-reactive Env, Gag, or Pol antigens were used to generate gene-specific components. Retained components were transformed using varimax rotation, to associate each antigen to one component (Figure 3).

Linear regression with robust standard errors was used to assess the association between HIV-1 specific antibody responses (using total and gene-specific components) and the following: CD4 count at baseline, viral load at baseline, change in CD4 count per year adjusted for baseline CD4 count, and change in viral load per year adjusted for baseline viral load. Logistic regression was used to assess the effects of HIV-1 specific antibody responses (using total and gene-specific components) on disease progression using CD4 count <350 cells/mm^3 or ART initiation as the disease progression endpoint. We also evaluated whether deworming treatment modified any associations examined with HIV-specific antibody responses. All interaction terms assessing this effect modification were not statistically significant. Statistical analyses were performed using Stata 11.2 (StataCorp).

Results:

Among the 92 individuals evaluated and analyzed, 76.1% were female and the mean age was 33.6 years. Most participants were married (56.5%), of low economic status, and relatively immunocompetent with a mean CD4 count and viral load of 515.8 cells/mm^3 and 4.01 $\log_{10}$ copies/mL, respectively. The study ended prior to 4 participants completing their 24 month study visit, making 18 months their final study visit. Nine of 92 participants began ART between baseline and endpoint. Evaluating change in CD4 and viral load prior to ART initiation from baseline to endpoint, the mean change in CD4 count was -108.2 cells/mm^3 per year and viral load was 0.02

log_{10} plasma RNA copies/mL per year. Among ART naïve participants, at baseline and endpoint there were 23 and 25 patients with CD4<350, respectively. Using a composite indicator of disease progression (CD4<350 or ART use), at baseline and endpoint there were 23 and 34 participants with progressive HIV-1 disease, respectively. Of the 88 individuals observed at 24 months, 25 had a CD4 count <350, 2 had started ART, and 7 had started ART as well as had a CD4 count <350 (Table 3).

We compared HIV-1-specific antibody responses at baseline among ART naïve individuals with more traditional markers of HIV-1 disease. The first 4 components of the total PCA accounted for 72% of the total variance of the 40 sero-reactive antigens. After orthogonal rotation, total components 1-4 accounted for 23%, 21%, 19% and 9% of the total variance, respectively. Two Env, 2 Gag, and 3 Pol-specific components were retained, accounting for 79%, 87%, and 77% of the total gene-specific variance, respectively. After rotation, each retained gene-specific component contained mostly 1 protein-specific antibody response, improving the interpretation of each component. Env1 (gp41: TM) and Env2 (gp120: SU) accounted for 56% and 23% of the total variance, respectively. Gag1 (p24: CA) and Gag2 (p17: MA) accounted for 47% and 40% of the total variance, respectively. Finally, Pol1 (p66; RT), Pol2 (IN), and Pol3 (p51: RT) accounted for 34%, 23%, and 19% of the total variance, respectively. HIV uninfected samples were also identically evaluated by protein microarray and demonstrated no sero-reactivity to HIV-specific antigens, demonstrating the specificity of the HIV-specific antibody responses (Figure 2).

Total and gene specific components were significantly associated with higher and lower viral load at baseline, indicated by the sign of the coefficient (Table 4). Total1 (RT p66, TM, V5, PR, p=0.023), Env1 (TM, p=0.007) and Pol1 (RT p66, p=0.028) were associated with greater viral load while Total2 (IN, RT p51, SU, MA, p=0.001), Total3 (CA, Nef, Rev, p=0.018), Env2 (SU, p=0.002), Gag1 (CA,

p=0.028), Gag2 (MA, p=0.004), and Pol2 (IN, p=0.006) were associated with lower viral loads. No total or gene specific components were significantly associated with CD4 count at baseline, though the components associated with higher viral loads had coefficients suggesting a relationship with lower CD4 counts. After baseline, Total3 (CA, Nef, Rev, p=0.012), Gag1 (CA, p=0.034), Gag2 (MA, p=0.037), and Pol2 (IN, p=0.063) were significantly associated with less decrease in CD4 counts from baseline to endpoint, adjusted for baseline CD4 measures and excluding time after ART among the subset who initiated ART. Additionally, when also adjusted for baseline viral load, the association between change in CD4 count and the following components was largely unchanged: Total3 (CA, Nef, Rev, p=0.024), Gag1 (CA, p=0.066), and Gag2 (MA, p=0.084). In contrast, no total or gene specific components were associated with change in viral load, comparing individuals with similar baseline viral load measures and no ART use.

We also compared HIV-1-specific antibody responses at baseline with disease progression (CD4<350 or ART initiation) at endpoint (Figure 4). Total3 was significantly associated with a 21% reduced odds of disease progression (CA, Nef, Rev, p=0.043). Among gene-specific components, there was a trend for decreased odds of disease progression for both Gag1 (CA, p=0.092) and Pol2 (IN, p=0.059), corresponding to a 21% and 29% reduced odds respectively.

Discussion:

Using principal components analysis, we were able to examine a broad array of HIV-specific antibody responses and to evaluate associations between complex patterns of antibody responses with disease progression. From our 40 sero-reactive antibody responses, we extracted 4 meaningful total antigen components that summarized 72% of the variance in the data. Further refining the PCA by gene specificity created 7 protein specific components that then could be

examined as more specific predictors of HIV disease progression. PCA in conjunction with protein microarray is a novel technique for exploring patterns of antibody responses within multivariate data.

The HIV-specific antibody responses demonstrated varying relationships with disease control, suggesting not all are equally effective, or involved in controlling the HIV virus. Among total sero-reactive components, Total1 (RT p66, TM, V5, PR) was associated with higher viral load, while Total2 (IN, RT p51, SU, MA) and Total3 (CA, Nef, Rev) were associated with lower viral loads at baseline. Over 12 months, Total3 was associated with smaller decreases in CD4 count and less likelihood of disease progression (CD4<350 or ART use). No associations with change in viral load were observed, as mean change in viral load was roughly zero. Relationships with total components highlighted patterns within the antibody responses. The collection of antibody responses in each component exhibit similar variation, but are not necessarily all individually associated with disease progression to a p-value of 0.05. Among protein-specific antibody responses, TM and RT p66 were associated with higher baseline viral loads, while SU, CA, MA, and IN were associated with lower viral loads. Over 12 months, antibody responses to CA and MA were significantly associated with smaller decreases in CD4 count, and antibody responses to CA and IN showed a trend for an association with reduced likelihood of disease progression though not below p=0.05.

We observed differences in the relationship between individual antigens and HIV-1 disease progression similar to other studies. In studies of Thai and US populations, investigators have observed significantly lower CA-specific antibody responses among rapid compared to slow progressors [89,90]. Here, CA and MA-specific responses were associated with lower baseline viral load and less CD4 decline over time. We also observed that higher Gag-specific IgG was associated with decreased progression independent of baseline viral load. Though Gag-specific antibodies

have little to no antiviral activity, they may be important in T-cell helper responses [84]. Additionally, over 12 months, higher CA, Nef, and Rev-specific antibody responses considered together (Total3) were significantly associated with reduced risk of disease progression. Anti-Nef and anti-Gag antibody responses have exhibited parallel relationships with disease progression before [91]. Efficient Nef-specific antibody dependent cellular cytotoxicity has been observed, and along with Nef's involvement in the evasion of host adaptive immunity, there may be a role for anti-Nef antibodies [92]. However, the involvement of anti-Rev antibody responses is less clear and may not be significantly associated with disease progression independent of CA and Nef.

Also unclear are the roles of anti-IN and anti-Env antibody responses in disease control. HIV-1 integrase (IN) enzyme integrates viral DNA into the host's genomic DNA, allowing the virus to establish a chronic infection. The enzyme is highly conserved and few mutations are seen among integrase inhibitor naïve patients [93]. Other studies detect anti-IN antibody responses during initial infection, but relationships with disease progression have not been shown [94]. Here we observed a trend for an association between greater anti-IN antibody responses and reduced disease progression, as well as less decline in CD4 count. Finally, previous studies of Env-specific IgG responses have shown mixed or no associations with disease progression [84,85]. In this analysis, greater anti-TM IgG was associated with higher viral load at baseline but not with subsequent changes in CD4 count, viral load, or disease progression at follow up. In contrast, greater anti-SU IgG was associated with lower baseline viral load, and the two responses were negatively correlated ($p<0.001$), demonstrating inverse relationships with HIV-1 disease.

Strengths of this analysis include the use of PCA to reduce a large dataset to a subset of created variables that account for most of the observed variation, allowing for the identification of patterns that can be explored and refined, reducing the number of comparisons made. The association of

collections of HIV-specific antibody responses with disease progression may have been missed if only individual antibody responses were considered. In addition, the inclusion of 92 individuals in the analysis is a larger sample size than prior studies of HIV-specific humoral responses and disease progression [84,85,89,90].

There were some limitations to this analysis. First, the study included samples collected over a 12 month period, which may be a short time frame of observation to adequately measure disease progression. Despite this, interesting antibody-specific associations were observed. Second, only 40 of the 143 antigens printed on the slide were sero-reactive. Finally, we did not examine the subclass or function of binding antibody responses or the role of conformational epitopes in this analysis. Identifying IgG subclass specificity or antiviral function would be important to further understand the mechanism behind antibody specific responses and disease control [83].

High-throughput immune profiling by protein microarray, along with PCA to reduce data dimensionality, is a feasible and straightforward approach to exploring complex immune responses, such as seen in HIV infection. Using this technique, our results suggest that viral load alone may not be driving HIV-specific IgG responses but alternatively antibody function. The associations demonstrated here warrant further examination to understand the mechanisms behind HIV-specific antibody responses in disease control. Future applications of this technique may include profiling immune responses of HIV co-infections, as defective antibody responses are characteristic of HIV progression.

CHAPTER 2. LOWER HIV-SPECIFIC ANTIBODY RESPONSES AND HIGHER VIRAL LOADS ARE ASSOCIATED WITH SCHISTOSOMIASIS CO-INFECTION BUT NOT OTHER HELMINTH SPECIES AMONG ART NAÏVE KENYAN ADULTS

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Abstract:

Considerable geographic overlap between HIV and helminth infections suggests that many of the 23.5 million Africans living with HIV may be co-infected with helminths. Immune suppression

induced by helminths may influence HIV disease. Using a nested study design among 100 HIV infected, ART naïve adults in Kenya, we examined the association of any helminth infection on HIV-specific antibody responses, as well as species-specific associations. Antibody response were clustered and analyzed by principal components analysis. HIV-infected adults with Schistosomiasis co-infection had significantly lower HIV-specific antibody responses (Comp 1, p=0.002; Comp 2, p=0.035) and higher viral loads than helminth uninfected individuals. These differences were not seen for other helminth species (ascaris, trichuris, or hookworm). These findings suggest that schistosomiasis may suppress humoral immune responses and increase HIV disease progression. Therefore, treatment for schistosomiasis may be useful to integrate with current HIV care.

Introduction:

Globally, 2 billion people are infected with at least one helminth species and considerable geographic overlap exists among HIV and helminth infections. Of the 22 million Africans estimated to be infected with HIV [17], many may be co-infected with at least one species of helminth [18].

Helminths induce immunosuppressive responses, creating ideal environments for chronic helminth infection [19,36]. Because of this induced immune suppression, helminths may have adverse effects on concurrent diseases, such as HIV infection. Studies suggest that treating helminth infections among HIV infected individuals may delay HIV progression, as measured by CD4 count and HIV viral load [4,43,95,96]. HIV infection also leads to immune dysregulation in global B cell function within the infected host [23]; with elevated IgG levels, or hypergammaglobulinemia [24]. Understanding whether helminths modify humoral immune responses to concurrent infection is important because HIV-specific antibody responses play a role in the control of HIV disease.

Using a nested cross-sectional design, we examine the association of helminth infection on HIV specific antibody responses. HIV antibody responses are measured by protein microarray and clustered using principal components analysis. We highlight species specific associations, as well as relationships due to any helminth infection.

Methods:

Study design

A cross-sectional analysis of helminth infected and uninfected individuals was conducted on 100 stored samples from the control arm of a large randomized controlled trial evaluating the effect of empiric deworming on markers of HIV-1 disease progression in Kenya [87]. Plasma samples were collected between February 2009 and July 2010. All individuals provided written informed consent to participate in the study. The trial was independently approved by the IRB at University of Washington and the Ethical Review Board of the Kenya Medical Research Institute. The parent study was registered as NCT00507221, http://clinicaltrials.gov.

Population

Study participants were enrolled from three sites in Kenya (Kisii Provincial Hospital, Kisumu District Hospital, and Kilifi District Hospital) who were older than 18, not pregnant, did not meet criteria for ART initiation based on Kenyan Ministry of Health guidelines, had not used ART in the past, and were willing and able to give informed consent. At study completion, participants were screened by microscopy for helminth infection and treated if found to be infected. From the 479 individuals in the control arm, 367 completed the study and were screened for helminths, with 57 being infected and 310 being uninfected. Those who started ART before the end of the study were

excluded, leaving 50 helminth infected individuals and 254 uninfected individuals, of which 50 were randomly selected using computer generated random sampling.

HIV-1 Microarray construction

HIV-specific antibody responses were measured in duplicate using protein microarray. The HIV-1 microarray was constructed by amplifying and expressing the open reading frames encoding 16 HIV-1 proteins and 13 gene fragments from each of the five major subtypes (A1, A2, B, C, D), or clades of HIV-1. Subsequently, the expressed proteins were printed onto nitrocellulose-coated slides. A total of 143 HIV-1 proteins or protein fragments from 5 virus clades and all 9 viral genes were printed on the arrays.

Full-length infectious molecular clones of HIV-1 were used as templates for polymerase chain reaction (PCR) to create each protein or peptide-encoding gene or gene fragment. The templates used were HIV-1 clones 92UG037, subtype A1; 94CY017, subtype A2; JR-CSF, subtype B; MJ4, subtype C and 94UG114, subtype D. All templates were obtained from the AIDS Research and Reference Reagent Program except JR-CSF (from Dr. Irvin Chen, UCLA). Primers were designed to contain twenty base pairs of homology to each ORF being amplified and thirty-three base pairs of homology to the plasmid vector, pXT7. The standard PCR cycle was 95^{o}C for 5 minutes followed by 39 cycles of 95^{o}C for 20 seconds, 50^{o}C for 30 seconds and 72^{o}C for 30 seconds for every 500 base pairs of product desired, followed by a final extension of 72^{o}C for 10 minutes. Amplification success was determined by PCR product visualization following agarose gel electrophoresis.

PCR products were inserted into the expression vector, by *in vivo* recombination as previously described [88]. Genes and gene fragments encoding HIV-1 proteins and protein fragments in pXT7 were expressed in an *E.coli*-based cell-free coupled *in vitro* transcription-translation (IVTT; Roche)

reaction, solubilized with Tween-20 and printed on nitrocellulose-coated FAST slides (GE-Schleicher and Schuell) using an Omni Grid 100 microarray printer. Microarrays were probed using human sera or control antibodies as previously described [88]. Microarrays were scanned using a ScanArray 4000 machine. QuantArray software was used to quantify the intensity of the spots on the chip. The signal intensity of the "No DNA" controls were averaged and used to subtract background reactivity from the unmanipulated raw data. Of the spotted proteins, 15 had signals greater than the average of "no DNA" control reactions plus 2.5 times the standard deviation, and were considered sero-reactive.

Data collection

Plasma samples were collected periodically throughout the study to monitor HIV disease, and demographic measures were collected at enrollment. CD4 count was measured every 6 months using a FACSCalibur (Becton Dickinson; Franklin Lakes, NJ, USA), and plasma HIV viral load was measured every 12 months using COBAS Amplicor assay (Roche Molecular Systems Inc, Branchburg, NJ, USA). Stool samples were collected at the final study visit for direct helminth microscopy and analyzed by a trained laboratory technologist using Kato-Katz and formol-ether concentration.

Statistical analysis

Principal components analysis (PCA) was used to reduce dimensionality of the HIV specific antibody responses. All 15 sero-reactive antigens were used to generate total antigen principal components, which base grouping on the variance of each antigen rather than any pre-specified criteria. Components were retained if at least 3 variables loaded, and the eigenvalue was greater than 2 or the proportion of variance explained was >5%. Retained components were transformed

using varimax rotation, to maximally associate each antigen to one component (Figure 1). Linear regression with robust standard errors was used to compare HIV specific antibody responses by helminth infection. Any infection was considered as well as infection by species. Student's t-test was used to compare participant characteristics by helminth infection, except CD4 and CD8 count, which were tested by rank sum test. Statistical analyses were performed using Stata 11.2 (StataCorp).

Results:

Among the 50 helminth infected individuals, 3 had ascaris, 19 had hookworm, 5 had trichuris, 15 had schistosomiasis, and 8 had mixed infections of at least 2 helminth species. Participants with and without helminth infection were similar in gender, viral load, CD4 count, CD8 count, white blood cells, red blood cells, hemoglobin, and hematocrit. Those with any helminth infection were significantly younger (30.2 vs. 34.7, p=0.02) and had lower BMI (21.4 vs. 23.0, p=0.02) compared to uninfected individuals. Those with ascaris infection were significantly younger (26.6 vs. 34.7, p=0.04) compared to uninfected individuals. Those with hookworm infection had significantly lower BMI (21.0 vs. 23.0, p=0.03) compared to uninfected individuals. Those with schistosomiasis infection were significantly younger (25.1 vs. 34.7, p<0.001) and had higher viral load (4.8 vs. 4.2, p=0.039) compared to uninfected individuals (Table 5).

We clustered HIV-1 specific antibody responses using unspecified PCA on all sero-reactive antibody responses. The first 2 components of the PCA accounted for 61% of the total variance. After orthogonal rotation, components 1 and 2 accounted for 33% and 27% of the total variance respectively. Component 1 represented most of the variance attributed to CA (capsid protein) and vpu (vpu protein) specific antibody responses, while component 2 represented most of the variance

attributed to gp41 (transmembrane protein, gp41) and IN (integrase protein) specific antibody responses (Figure 5).

We compared HIV-specific antibody response by helminth infection. Comparing individuals with any helminth infection to uninfected individuals, there was no difference in HIV-1 specific antibody responses. However, when helminth species were considered individually, those with schistosomiasis infection had significantly lower HIV specific antibody responses to CA and vpu proteins (component 1: -1.51, p=002), as well as significantly lower HIV specific antibody responses to gp41 and IN proteins (component 2: -0.97, p=0.035), compared to uninfected individuals. There were no differences in HIV specific antibody responses comparing the other helminth species to helminth uninfected individuals (Table 6).

Discussion:

In this study, we compared humoral responses to HIV antigens in HIV infected adults co-infected with helminths versus not. Overall, those with helminth infection did not differ in HIV specific antibody responses than those with helminth responses. However, individuals with schistosomiasis infection had lower HIV-specific antibody levels than those without helminth infections. We used a protein microarray with principal components analysis, HIV specific antibody responses clustered into 2 predominant components, accounting for 61% of the total variance in HIV antibody responses. While both HIV components demonstrated an association with schistosomiasis infection, the magnitude of antibody responses to CA and vpu proteins was greater than that to antibody responses for gp41 and IN proteins.

Some HIV specific antibody responses may play a role in delaying HIV progression. In a previous evaluation of this cohort, we found that greater antibody responses to CA, MA, and IN proteins were

associated with a slower decline in CD4 count over 12 months [see chapter 2]. Lower antibody responses to these HIV antigens may represent decreased control of HIV infection. Additionally, those with schistosomiasis also had higher viral loads compared to helminth uninfected individuals, which also correlates with more rapid progression of HIV disease.

Our study suggests that helminth infections differ in their influence on HIV progression. Schistosomiasis is a systemic infection which may result in more tissue destruction from migrating eggs, and more immune activation. Consistent with our findings, a meta-analysis examining species specific treatment effects of helminth and HIV co-infection identified a trend towards an association between schistosomiasis treatment and a decrease in HIV viral load, which was not seen with other helminth species [95].

A limitation of this study is its cross sectional design. How HIV specific antibody responses and viral loads change with schistosomiasis infection is unclear from this study. The number of individuals with each helminth species was small with minimal power to evaluate helminths other than schistosomiasis and hookworm; however, a significant difference in HIV specific antibody responses was still identified for shistosomiasis. Additionally, the duration and intensity of helminth infections were unknown. Finally, there is not a clear HIV specific humoral response that is a definitive immune correlate of progression.

Schistosomiasis may suppress an individual's humoral immune response, decreasing antibody responses to concurrent HIV infection and increasing HIV disease progression. If this is the case, treatment for schistosomiasis may be a cheap and easy intervention to improve health outcomes associated with concurrent HIV disease.

CHAPTER 3. HELMINTH INFECTION IS NOT ASSOCIATED WITH SPONTANEOUS ANTIBODY RESPONSES TO PREVIOUSLY ADMINISTERED MEASLES AND TETANUS VACCINES AMONG HIV INFECTED, ART NAÏVE KENYAN ADULTS

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Abstract:

Morbidity and mortality due to vaccine preventable illness account for a substantial portion of the global burden of disease. Both HIV and helminth infections compromise vaccine immune responses and many regions with high HIV prevalence also have high helminth prevalence. To determine whether helminths alter vaccine responses among HIV infected individuals, we conducted nested serologic studies within two cohorts of HIV infected adults. From the control arm of a previously conducted randomized trial (RCT), we compared antibody responses to measles and tetanus in 50

helminth-infected and 50 uninfected individuals. We also compared measles and tetanus antibody responses in Ascaris-infected adults receiving albendazole (16) vs. placebo (19) enrolled in a separate RCT. In both cohorts, over 70% of participants had measles antibody levels above the protective threshold, and all participants had tetanus responses above the protective threshold. Prevalence of measles response and levels of tetanus antibody response were similar between helminth-infected and uninfected individuals and did not differ by helminth species. In the ascaris-infected cohort, change in measles and tetanus responses did not differ between those who received anthelminthics vs. placebo. Although we did not detect an impact of helminth infection on spontaneous vaccine-specific antibody responses among HIV infected adults, it is possible that helminth infection may impact such responses in HIV infected children, in whom helminths are more common and vaccination is more recent.

Introduction:

Helminths are responsible for considerable numbers of infections around the world. Hookworm and Schistosoma species alone, cause over 500 million and 200 million infections, respectively [97]. These types of infections are associated with poverty, occurring predominately in developing countries where sanitation, hygiene, and access to proper health services, such as immunizations, may also be poor [15].

Considerable geographic overlap also exists among HIV and helminth infected individuals. Of the 22 million Africans estimated to be infected with HIV [17], many are likely co-infected with at least one species of helminth [18]. Helminths induce immunosuppressive responses in order to survive in the infected host long term, and helminth co-infection may have consequences on concurrent HIV

disease, as deworming has been shown to decrease HIV-1 viral load and may impact other markers of HIV disease progression, including CD4 count [4,15].

There may also be an impact of helminths on bystander immune responses such as vaccine responses, which are also diminished in individuals with HIV infection [14,98]. Vaccine preventable illnesses are of great concern in children; however, measles continues to occur in adults, leading to greater complications among older and immunosuppressed individuals, as well as hindering eradication efforts [99,100].

We sought to determine whether helminth infection alters responses to measles and tetanus vaccines among HIV-1 infected, ART naïve adults in Kenya. Using two nested study designs, we examined whether infection with any species of helminth is associated with unstimulated measles and tetanus antibody responses, as well as whether deworming modulates those vaccine-specific responses 3 months later, among HIV and Ascaris co-infected individuals.

Methods:

We conducted serologic assays for measles and tetanus on repository specimens from two previously accrued cohorts involving HIV infected adults.

Study 1: Helminth infected compared to uninfected, HIV positive individuals

Study design

A cross-sectional analysis of helminth infected and uninfected individuals was conducted on 100 stored samples from the control arm of a large randomized controlled trial evaluating the effect of empiric deworming on markers of HIV-1 disease progression in Kenya [101]. Plasma samples were collected between February 2009 and July 2010. All individuals provided written informed consent

to participate in the study. The trial was independently approved by the IRB at University of Washington and the Ethical Review Board of the Kenya Medical Research Institute. The trial was registered as NCT00507221 at http://clinicaltrials.gov.

Population

Study participants were enrolled from 3 sites in Kenya (Kisii Provincial Hospital, Kisumu District Hospital, and Kilifi District Hospital) who were older than 18, not pregnant, did not meet criteria for ART initiation based on Kenyan Ministry of Health guidelines, had not used ART in the past, and were willing and able to give informed consent. At study completion (2 years after enrollment), participants were screened by microscopy for helminth infection and treated if found to be infected. From the 479 individuals in the control arm, 367 completed the study and were screened for helminths, with 57 helminth-infected and 310 helminth-uninfected. Those who started ART before the end of the study were excluded, leaving 50 helminth-infected individuals and 254 uninfected individuals. The nested serologic study included all of the 50 helminth-infected individuals. Fifty uninfected individuals were selected using computer-generated random selection.

Data collection and statistical analysis

Demographic measures were collected at enrollment and plasma samples were collected periodically throughout the study to monitor HIV disease. CD4 count was measured every 6 months using a FACSCalibur (Becton Dickinson; Franklin Lakes, NJ, USA), and plasma HIV viral load was measured every 12 months using COBAS Amplicor assay (Roche Molecular Systems Inc, Branchburg, NJ, USA). Plasma specimens from the final visit (24 months) concurrent with stool helminth testing were used for serologic assays. Stool samples were assessed by direct helminth microscopy and analyzed by a trained laboratory technologist using Kato-Katz and formol-ether concentration. Vaccine-specific antibody responses were measured in duplicate using commercially

available ELISA kits (Diagnostic Automation, Inc., Calabasas, CA, Measles: Cat#1408Z; Tetanus: Cat#8900Z). Positive, equivocal, or negative measles responses were dichotomized, including equivocal as negative, and compared by chi-squared tests. Tetanus responses were log transformed and the association between tetanus response and helminth infection was evaluated by Student's t-test. This study had 80% power to detect a 31% difference in measles positivity between the helminth infected and uninfected groups, and a mean difference in log10 tetanus response of 0.28 between the helminth infected and uninfected groups. Statistical analyses were performed using Stata 11.2 (StataCorp).

Study 2: Dewormed compared to placebo treated, Ascaris and HIV co-infected individuals

Study design

A nested cohort study of dewormed and placebo treated Ascaris and HIV-co-infected individuals was conducted on 35 stored samples from a randomized controlled trial evaluating the short-term impact of deworming on markers of HIV-1 disease progression in Kenya [43]. Plasma samples were collected between March 2006 and June 2007. All individuals provided written informed consent to participate in the study. The trial was independently approved by the IRB at University of Washington and the Ethical Review Board of the Kenya Medical Research Institute. The trial was registered as NCT00130910 at http://clinicaltrials.gov.

Population

Study participants were enrolled from 10 sites across Kenya, who were older than 18, not pregnant, did not meet criteria for ART initiation based on Kenyan Ministry of Health guidelines, had not used ART in the past, and were willing and able to give informed consent. Individuals infected with a

soil-transmitted helminth treatable by albendazole were randomized to deworming treatment, consisting of 400mg albendazole given once daily for 3 consecutive days, or placebo with the first dose observed in the clinic, and asked to return in 3 months. At follow-up all participants with helminth infection were given deworming treatment. From the 208 co-infected individuals enrolled in the study, 54 were Ascaris co-infected, and of those, 35 had sufficient stored sample for analysis.

Data collection and statistical analysis

Demographic measures were collected at enrollment and plasma samples were collected at baseline and follow-up to monitor HIV-1 disease. CD4 counts were determined using Multiset software on a FACSCalibur machine (Becton Dickinson, USA). Plasma HIV-1 viral loads were quantified using the Gen-Probe HIV-1 viral load assay. Stool samples were processed and evaluated using wet-preparation, Kato-Katz and formol-ether concentration techniques by an experienced laboratory technician. Vaccine-specific antibody responses were measured in duplicate using commercially available ELISA kits (Diagnostic Automation, Inc., Calabasas, CA, Measles: Cat#1408Z; Tetanus: Cat#8900Z). Positive, equivocal, or negative measles responses were dichotomized, including equivocal as negative, and compared by chi-squared tests. Tetanus responses were log transformed and the association between deworming treatment and tetanus response at 3 months, adjusted for baseline tetanus responses was evaluated using linear regression with robust standard errors. This study had 80% power to detect a 34% difference in increased measles positivity between dewormed and placebo groups, and an 83% difference in tetanus response at 3 months between dewormed and placebo groups. Statistical analyses were performed using Stata 11.2 (StataCorp).

Results:

Comparison of measles and tetanus responses in helminth-infected to helminth-uninfected HIV-infected adults

The 50 HIV-infected individuals with helminths and 50 without helminths were comparable in gender, viral load, and CD4 count. However, those with helminth infections were slightly younger (30.2 vs. 34.7, p=0.02) than uninfected individuals. Both groups were comparable in education, occupation, and number of children in the household. Those with helminth infections were more likely to use environmental water sources and pit latrines than those without helminth infections. Few individuals had piped water or flush toilets in their homes (Table 7).

Among individuals without helminth infection, 72% were seropositive for measles, which was lower than prevalence of measles seropositivity among those with helminth infection (82%). Rates of measles seropositivity ranged from 74% to 88% by helminth species. There were no significant differences in measles positivity by any helminth infection, nor by species-specific helminth infection (Figure 6). Log transformed tetanus responses were examined by any helminth infection, as well as species-specific helminth infection. All individuals had a tetanus response above the protective threshold of 0.01 IU/mL [14]. Comparing individuals with any helminth infection to helminth uninfected individuals, there was no difference in mean log10 tetanus response (-0.133 IU/mL vs. -0.190 IU/mL, p=0.56). By helminth species individually, there also was no difference between helminth infected and uninfected in log10 tetanus response (Figure 6). Though age was associated

with helminth infection and tetanus response, adjusting for age did not change these results.

Comparison of measles and tetanus responses in dewormed to placebo treated, Ascaris and HIV co-infected adults

Among the 35 Ascaris and HIV co-infected individuals, dewormed and placebo recipients were comparable at baseline in gender, age, viral load and CD4 count before treatment. Education was comparable between the treatment groups, but the dewormed group had more unemployed individuals, fewer farmers, and more children in the household. More individuals in the placebo group used environmental water sources and pit latrines outside the home. Again, few people had piped water and flush toilets in the home (Table 7). However, none of the differences were statistically significant.

Among HIV and Ascaris co-infected individuals, 3 individuals of the 16 in the placebo group, changed from positive to negative, while 1 of 14 in the dewormed group changed from negative to positive (p=0.15). There were no differences in log10 tetanus response at 3 months both with and without adjusting for baseline tetanus responses, between dewormed or placebo treated individuals (adjusted, p=0.39). (Table 8)

Discussion:

In this study, helminth infection was not associated with lower measles and tetanus IgG responses among HIV infected adults in Kenya who were not yet antiretroviral treatment (ART) eligible. Most individuals had measles responses and all had protective levels of tetanus antibody suggesting

preservation of vaccine responses despite both helminth and untreated HIV infection. We did not detect a difference in vaccine-specific antibody responses by helminth infection or by species-specific helminth infection. We also evaluated whether deworming altered vaccine-specific responses among HIV and Ascaris co-infected individuals. Measles responses did not increase following deworming compared to placebo in ascaris-infected individuals. In addition, tetanus antibody levels were universally above the threshold of protection and levels of tetanus antibodies did not differ between helminth infected and uninfected individuals or between dewormed vs. placebo recipients.

Prevalence of measles seropositivity was greater than 70% in our study in both cohorts. This prevalence was lower than has been observed in previous studies among HIV infected adults in the UK and Mexico, in which prevalence of measles seropositivity was 93% and 76%, respectively [14,102]. While our study suggests that some of these adults may benefit from measles bosster vaccines, most HIV-helminth co-infected individuals had adequate protection despite both infections.

We investigated the association between unstimulated vaccine-specific antibody responses and helminth infection. While memory B cells do not actively secrete antibodies until triggered, plasma cells continually secrete antibody without further stimulation [54]. Additionally, antibodies specific to many childhood vaccines are detectable in plasma from adults who received vaccinations in early childhood [54,56]. Among healthy individuals, the half-life of tetanus specific serum antibody production is approximately 11 years, while for measles it is may be maintained for life [56]. Our study suggests that plasma cell secretion in these individuals persists despite large systemic immune perturbations due to HIV and helminth infection. This study cannot speak to the impact of helminths on stimulated immune cells such as memory B cells.

Our results do not exclude a possible association between helminths and responses to new vaccinations or vaccine efficacy, such as preventing infection when challenged. Even without circulating antibodies, vaccine-specific memory B cells can provide protective immunity following infection [55]. Studies of post-vaccination responses to BCG and an oral cholera vaccine among helminth infected individuals observed improved vaccine responses following albendazole versus placebo prior to vaccination [9,11]. Additionally, post-vaccination antibody responses to tetanus were lower in Onchocerciasis infected compared to helminth uninfected individuals [103].

We did not observe that deworming led to enhanced vaccine-specific antibody responses. In another study looking at helminths and antibody responses to concurrent HIV infection, individuals infected with schistosomiasis had lower HIV specific antibody responses than helminth uninfected individuals [CITE]. Additionally, spontaneous seroconversion to measles and tetanus were detected 6 months after ART among HIV infected children, underscoring that HIV effects likely outweigh helminth effects on vaccine response [104]. The deworming study had a small sample size and limited power to exclude anything but very large differences.

To our knowledge, other studies have not evaluated the association between helminths and vaccine responses among HIV infected adults. We used two nested cohort studies to evaluate whether helminth infection or treatment is associated with diminished measles and tetanus specific antibody responses. Limitations of this study include power to detect only large differences in vaccine responses, inability to evaluate appropriate recall of vaccine responses, and a short duration of follow-up.

Though helminth infection was not shown to be associated with spontaneous vaccine-specific antibody responses to measles and tetanus in HIV-infected adults, there may be different effects

with other vaccines. In addition, it remains important to evaluate helminth effects on vaccine responses in HIV-infected children, who may receive vaccines during helminth infection, and are more likely to harbor helminth infections and to have higher worm burden. Further studies in children will be useful to discern the influence of helminths and deworming, particularly among HIV-infected children who could be easily dewormed through HIV care and treatment [105].

CHAPTER 4. INTEGRATION OF DEWORMING INTO HIV CARE AND TREATMENT: A NEGLECTED OPPORTUNITY

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Viewpoint paper, published: [105]

In sub-Saharan Africa, where two-thirds of all HIV infected individuals reside, many are now aware of their HIV infection status and millions are receiving antiretroviral therapy. In many countries in the region, new infections are declining and individuals are living longer with treatment to control their HIV infection. The infrastructure behind HIV care and treatment is vital in addressing the HIV pandemic, and similarly could be utilized to address and promote other health issues specific to persons living with HIV.

An estimated 2.1 million children are infected with HIV in sub-Saharan Africa, where multiple helminth species are also endemic [106]. Likely half of these children are co-infected with helminths [107]. The most recognized consequences of helminth infection in children include anemia, malnutrition, and impaired cognitive development, which are independent risk factors for death among HIV-infected children. Standard treatment of soil-transmitted helminth infection

entails a single 400mg dose of albendazole [15], making routine deworming of children a simple intervention that safely and affordably prevents the adverse effects of chronic helminth infection. Deworming HIV infected children, specifically, may have a substantial impact on child health through the synergistic effects of improved nutritional status, greater control of other infectious diseases, and increased vaccine responsiveness, and therefore, should be provided though HIV care services [15].

Helminth infection in HIV infected children may impact host responses to infectious diseases and immunizations, indirectly through the pathway of malnutrition, as well as directly through immunologic mechanisms. Beyond nutritional deficits, helminths also induce immunosuppressive responses, creating an ideal environment for chronic helminth infection, and inhibiting the host's ability to control other diseases such as HIV [19,36]. Clinical studies suggest that deworming HIV infected individuals may delay HIV progression, as measured by CD4 count and HIV viral load [4,43,95,96]. It is plausible that in addition to improving nutritional status, eliminating helminths in infected individuals may directly impact control of other infectious diseases such as HIV.

Helminth infection may also undermine the benefits of childhood immunizations, through malnutrition, and also by diminishing immune responses to vaccines, both at the time of vaccination and at disease exposure. Population-level data shows that regional variations in vaccine efficacy correlate with variations in the prevalence of enteric pathogens [61]. For example, rotavirus vaccine efficacy may be 50% higher in developed countries compared to Africa and Asia [62]. Polio eradication efforts have also been challenged by diminished efficacy of the oral polio vaccine in India as compared to the rest of the world [63]. While the distribution of soil-transmitted helminths represents only one of several factors contributing to these regional variations in vaccine

responsiveness, it is a factor which can be easily targeted and controlled through routine deworming [65].

Individual-level evidence also suggests that helminth infection impacts immunologic responses to vaccines. Experimental human and animal studies have shown deworming before immunization increases protective antibody titers, while decreasing immuno-regulatory cytokines [6,7,10]. Children who failed to respond to oral poliovirus vaccination were 25% (p=0.04) more likely to harbor infections with intestinal parasites than vaccine responders [67]. Additionally, children with ascariasis who received albendazole prior to receiving oral cholera vaccine were 88% (p=0.06) more likely to seroconvert than children who were not dewormed [9]. Interactions that diminish responses to vaccines at the time of vaccination may also diminish immune recall of vaccines at the time of disease exposure. As HIV-infected children are more susceptible to vaccine preventable illness and death than other children [58], even after the introduction of anti-retroviral therapy [12], deworming HIV infected children may have a measurable impact on vaccine preventable infections.

The World Health Organization (WHO) recommends annual or bi-annual school-based deworming as a cost-effective strategy to diminish the consequences of chronic helminth infection. Deworming could also be considered part of the nutritional care package for HIV-infected children, to reduce the consequences of malnutrition and anemia in HIV. Incorporating deworming into routine HIV care and treatment is an ideal way to improve the nutritional health of HIV infected children, and may provide additional benefits. This may be particularly beneficial for children under five years of age, who represent 10-20% of the 2 billion people infected with helminths worldwide [108]. Annual deworming of preschool-age children is safe and highly effective in reducing parasite prevalence and intensity, malnutrition, and risk of stunting, but a formal policy does not yet exist to

target this age group [108,109]. Because children are infected and often diagnosed with HIV while very young, preschool-aged children can easily be dewormed in HIV clinics, along with siblings to reduce the occurrence of reinfection.

Despite WHO recommendations, school-based implementation is not universal and many helminth-infected school-age children go untreated. Children who are sick or otherwise unable to attend school may miss school-based interventions, leading to more illness and absenteeism. Children with HIV may also be less likely to receive other health services. For example, HIV infected children are less likely to receive complete vaccination series compared to uninfected children [110]. HIV care centers are an important, and highly accessed, point of serial contact for HIV-infected children and their families [111]. However, at present they often provide a narrow range of services. In addition to integrating deworming into current HIV treatment, the integration of other necessary childhood health interventions including vitamin supplementation, immunizations, safe drinking water (through home water filtration), and insecticide-treated bed nets, may further reduce HIV-related morbidity and mortality among these children [111].

Challenges may exist in coupling other health interventions to HIV care, but the potential benefits warrant consideration. Deworming both in schools and HIV clinics is likely justified by the high rates of recurrent infection in children and the low cost of the intervention. Finally, little evidence exists on the impact of deworming in HIV infected children, highlighting a need for more rigorous studies. These studies should investigate the effects of helminth infection on responses to immunizations, the potential interactions between antihelminthics and HIV treatment, the optimal timing of deworming around both immunizations and ART initiation, and the impact of deworming on incidence of vaccine preventable infections.

The benefits of treating and preventing helminth infections in HIV infected children may go beyond the improved nutritional status and cognitive development observed in all children, to also include improved responses to immunizations and control of other infectious diseases. Enhanced control of neglected infectious diseases, such as helminth infections, through existing HIV care and treatment programs, may further reduce childhood morbidity and mortality in this vulnerable population.

CONCLUSIONS

Prior to this study, an RCT examining the association of deworming compared to placebo with markers of HIV disease observed that deworming was associated with significantly higher CD4 counts among Ascaris infected individuals after 3 months of follow-up and a trend for lower HIV viral load among HIV infected, ART naïve adults [43]. There were no differences seen for other helminth species, however these outcomes suggest improved HIV disease. Additionally, among these Ascaris and HIV co-infected individuals, deworming was associated with significantly decreased plasma IL-10 levels [19], a helminth-induced immunosuppressive cytokine that may inhibit the host's control of HIV infection. If deworming improves HIV disease among helminth and HIV co-infected individuals, deworming may be a highly cost-effective intervention to slow HIV progression and allow individuals to delay initiation of antiretroviral therapy, which can be expensive, cumbersome, and have adverse side effects. Helminth infection is routinely managed by empiric treatment because diagnosis is labor intensive and costly, while deworming is safe, effective and cheap.

To evaluate whether empiric deworming was associated with delaying HIV disease progression a second RCT was conducted among HIV infected, ART naïve adults in Kenya. The results of the trial showed no association of empiric deworming with either time to a CD4 count <350, or a composite endpoint of CD4 count <350, ART initiation, or non-traumatic death [101]. These results suggest there may not be a clinically meaningful effect of empiric deworming on delaying HIV disease progression in adults living in helminth endemic areas. However, helminths have a biologic effect on their infected host, and this may impact in subtle ways how the host responds to concurrent infections, as well as immunizations.

This study aimed to explore whether helminth infections impact host humoral immunity to concurrent HIV infection and previously administered measles and tetanus vaccines. Humoral immune responses such as the generation of antigen-specific antibody responses are integral in protecting against infectious organisms. Successful vaccination is determined by a vaccine-specific antibody concentration above the protective threshold. Here, we measured vaccine-specific antibody responses using commercially available ELISAs. In order to assess HIV-specific antibody responses, methods were developed to identify clusters of HIV antibody responses that were associated with disease progression. Protein microarray and principal components analysis were combined to evaluate antibody responses to a range of HIV antigens and reduce those robust antibody responses to a meaningful grouping that related to HIV disease progression. At baseline, greater antibody responses to the transmembrane glycoprotein (TM) and reverse transcriptase (RT) were associated with higher viral loads, while responses to the surface glycoprotein (SU), capsid (CA), matrix (MA), and integrase (IN) proteins were associated with lower viral loads. Over 12 months greater antibody responses were associated with smaller decreases in CD4 count (CA, MA, IN), and reduced likelihood of disease progression (CA, IN). Additionally, associations observed among collections of antibody responses paralleled protein-specific responses.

To determine whether helminths alter HIV or vaccine-specific antibody responses among HIV infected individuals, we conducted nested serologic studies within the two cohorts described above. Helminth infected and uninfected individuals were identified at study completion from the control arm of the RCT evaluating the impact of empiric deworming on HIV disease progression. The prevalence of HIV and vaccine specific antibody responses was compared by any helminth infection and species-specific infection. The prevalence of HIV specific antibody responses was similar between individuals with any helminth infection compared to helminth uninfected

individuals. However, those with schistosomiasis infection had significantly lower HIV specific antibody responses (predominately CA, vpu, gp41, and IN), compared to helminth uninfected adults. Lower antibody responses to CA and IN indicated worse HIV disease progression in our previous study, suggesting Schistosomes may impact concurrent HIV disease. Comparing any helminth infection or species specific infection to helminth uninfected individuals, the prevalence of measles and tetanus antibody responses were similarly high in all groups. Interesting, antibody responses to HIV antigens, which HIV infected individuals are continually exposed to, were depressed by helminths, but not antibody responses to vaccine antigens, which would not be circulating in the body unless infected. The effect of helminths may be more on memory B cells, which secrete antibodies when triggered, and not on plasma cells, which secrete antibodies continually without further stimulation.

Changes in HIV and vaccine specific antibody responses were evaluated among dewormed or placebo treated, Ascaris and HIV co-infected adults after 3 months of follow-up. HIV specific antibody responses were comparable 3 months after deworming or placebo treatment (data not shown). While sero-positivity to measles changed from negative to positive for one dewormed individual, and from positive to negative for 3 placebo treated individuals, these changes in measles antibody responses were not statistically different. Additionally, changes in tetanus antibody responses were comparable between treatment groups. The lack of an effect of deworming on humoral responses may be due to low sample size (35) or too short of follow-up (3 months). Helminths impact the infected host in the form of chronic infections, so the immediate removal of worms may not reverse induced immune changes.

Ethical considerations limit researcher's ability to thoroughly study the biologic effects of human helminth infection. Deworming is known to be beneficial, both in infected individuals and in

individuals of unknown infection status. The standard of care for helminthiasis in children is empiric deworming, making the withholding or delaying of treatment in a randomized manner unacceptable. Adults, on the other hand, are not routinely treated unless diagnosed with a helminth infection, at which time withholding or delaying treatment would also be unacceptable. With the evidence currently available, the potential for a benefit of deworming on concurrent infections and vaccinations encourages mass treatment for all in endemic areas.

Future research should be in the form of community based implementation studies. Measuring the impact of community wide, mass deworming on multiple outcomes including concurrent diseases such as HIV, and new and previously administered immunizations would add to our understanding of human helminth infection. Stepwise inclusion of one community per time interval would allow comparison of mass dewormed and not yet dewormed communities at multiple time points. In the absence of vaccines against helminths, improvements in sanitation and hygiene, as well as regular deworming treatment are required to decrease the global impact of helminth infections. This opportunity for operational research should not be wasted, as the lessons potentially learned may not be gained otherwise.

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Table 1. Contrasting host immune responses to helminth and HIV infection.

HIV	**Helminth**
Chronic infection, causes immune dysfunction and death	Chronic infection, causes indirect effects on health through malnutrition, mostly asymptomatic
Intracellular, infects many different cell types	Extracellular, long-lived
Immune activation, eventually leading to immune suppression	Immune suppression
Th1 response and CTL for viral control, Th17 depletion and T reg increase with disease progression	Th2-like response, T regs
Replication through cell machinery, rapid mutation through high replication	Does not replicate in host, intensity of infection increases with reinfection
CD4+ Tcell depletion, CD8+ Tcell expansion, type I IFN, IL-7	IL-4, IL-5, IL-13, IL-10, TGF-b, IgE, eosinophils, mast cells
Innate immunity important in acute infection, adaptive response more important in chronic infection	Innate immunity important in both acute and chronic infection, adaptive immunity may be somewhat involved in chronic infection

Table 2. Immunomodulatory products of helminths.

Product	**Helminth**	**Mechanism of action**
Lysophospha-tidylserine	*S. mansoni*	TLR2-dependent activation of DCs promotes T_{Reg} cell development
Phosphatidyl-serine	*S. mansoni* and *A. lumbricoides*	TLR2-dependent activation of DCs promotes T_H 2-type anti-inflammatory responses
LNFPIII	*S. mansoni*	Production of IL-10 and prostaglandin E_2 by B-1 B cells; TLR4- and C-type lectin-dependent activation of APCs promotes T_H 2-type anti-inflammatory responses
ES62	*A. viteae*	Inhibition of B-2 B cell proliferation and induction of B-1 B cell-dependent IL-10 secretion; TLR4-dependent activation of APCs promotes T_H 2-type anti-inflammatory responses; TLR4-dependent inhibition of mast cell degranulation and inflammatory mediator production
dsRNA	*S. mansoni*	Modulates DC function, through TLR3, to suppress T_H2 cell priming of ovalbumin-specific T cells
Cathepsin cysteine proteases	*S. mansoni* and *F. hepatica*	Inhibits LPS-induced nitric oxide, IL-6, IL-12 and TNF production by macrophages by inhibition of TRIF signalling through endosomal degradation of TLR3
smCKBP	*S. mansoni*	Blocks CXCL8-induced migration and infiltration of neutrophils in a mouse air pouch model and in a chemotaxis model
IPSE	*S. mansoni*	IgE-binding factor that induces IL-4 production from basophils
Omega-1	*S. mansoni*	T2 ribonuclease that matures DCs to prime T_H2 cell responses in a MYD88- and TRIF-independent manner
Peroxiredoxin	*F. hepatica* and *S. mansoni*	Promotes T_H2 cell responses through induction of alternatively activated macrophages
Cystatin	*A. viteae* and *O. volvulus*	Inhibition of T cell responses by macrophage-derived IL-10
Calreticulin	*H. polygyrus*	Promotes T_H2 cell-responses by interacting with scavenger receptor A
DiAg	*D. immitis*	CD40-dependent polyclonal IgE production

* Adapted from Harnett 2010 [39]

Table 3. Patient characteristics at enrollment (n=92).

Characteristics at Enrollment	**N (%) or Mean (SD)**	
Female	70 (76.1%)	
Age at enrollment	33.6 (8.2)	
Deworming Treatment Arm	46 (50.0%)	
Clinic Location		
Kisii	44 (47.8%)	
Kisumu	48 (52.2%)	
Marital status		
Single	14 (15.2%)	
Married	52 (56.5%)	
Divorced/Separated	8 (8.7%)	
Widowed	18 (19.6%)	
Education (highest completed)		
None	5	
Less than primary	14	
Primary school	36	
Secondary or Post-secondary training	37	
Has an income generating occupation		
None	12 (13.0%)	
Business/Employed by others	8 (8.7%)	
Business self employed	27 (29.3%)	
Casual laborer	8 (8.7%)	
Farmer	22 (23.9%)	
Professional (teacher, lawyer, etc)	15 (16.3%)	
Income per month (1 missing)		
<2000	47 (51.1%)	
2,000 – 4,999	18 (19.6%)	
5,000 – 9,999	11 (12.0%)	
>10,000	15 (16.3%)	
Health measures among ART naïve	**Month 12 (n=92)**	**Month 24 (n=79)**
BMI (kg/m^2)	22.6 (3.8)	22.7 (4.4) [n=76]
CD4 count ($cells/mm^3$)	515.8 (232.6)	438.6 (176.7)
Viral load (log_{10} copies/mL)	4.01 (0.97)	3.94 (0.95)
Change in CD4 per year, 12-24M [n=89] [1]	-108.2 (169.3)	
Change in log_{10} viral load per year, 12-24M [n=79] [1]	0.02 (0.56)	
CD4 count <350	23	25
Disease Progression (CD4 count <350 or ART initiation)	23	34[2]

[1] Slope is change in CD4 or viral load before ART initiation, for those who started ART

[2] 25 CD4 <350, 2 ART use, 7 ART use plus CD4 <350 (n=88)

Table 4. Association between HIV-1-specific antibody responses and CD4 count or log_{10} viral load.

	CD4 count at 12 Months [n=92] [1]			**Log_{10} viral load at 12 Months** [n=92] [1]		
	Coefficient	P-value	95% CI	Coefficient	P-value	95% CI
Total antigens 1 (RT p66, TM, V5, PR)	-9.861	0.229	-26.055, 6.331	0.078	0.023	0.011, 0.145
Total antigens 2 (IN, RT p51, SU, MA)	11.073	0.364	-13.052, 35.198	-0.102	0.001	-0.164, -0.040
Total antigens 3 (CA, nef, rev)	14.312	0.149	-5.221, 33.844	-0.087	0.018	-0.159, -0.015
Total antigens 4 (vpu, vpr)	-12.155	0.324	-36.500, 12.191	-0.053	0.289	-0.151, 0.045
Env antigens 1 (TM, V5)	-12.474	0.242	-33.523, 8.574	0.117	0.007	0.032, 0.201
Env antigens 2 (SU)	19.456	0.438	-30.149, 69.062	-0.181	0.002	-0.296, -0.067
Gag antigens 1 (CA)	22.490	0.080	-2.717, 47.697	-0.104	0.028	-0.197, -0.011
Gag antigens 2 (MA)	12.599	0.508	-25.023, 50.221	-0.140	0.004	-0.233, -0.046
Pol antigens 1 (RT p66)	-15.221	0.174	-37.278, 6.836	0.105	0.028	0.011, 0.199
Pol antigens 2 (IN)	16.892	0.267	-13.122, 46.905	-0.148	0.006	-0.253, -0.043
Pol antigens 3 (RT p51)	16.069	0.377	-19.882, 52.021	-0.075	0.184	-0.187, 0.037
	Change in CD4 count per year after 12 months (slope) [n=89] [2]			**Change in Log_{10} viral load per year after 12 months (slope)** [n=79] [2]		
Total antigens 1 (RT p66, TM, V5, PR)	-1.241	0.786	-10.314, 7.832	0.016	0.473	-0.029, 0.061
Total antigens 2 (IN, RT p51, SU, MA)	8.148	0.185	-3.986, 20.282	-0.011	0.574	-0.051, 0.029
Total antigens 3 (CA, nef, rev)	11.347	0.012	2.562, 20.131	-0.001	0.965	-0.051, 0.049
Total antigens 4 (vpu, vpr)	3.863	0.628	-11.929, 19.656	0.035	0.316	-0.035, 0.106
Env antigens 1 (TM, V5)	-2.930	0.633	-15.077, 9.218	0.027	0.328	-0.028, 0.082
Env antigens 2 (SU)	13.674	0.226	-8.632, 35.979	-0.014	0.694	-0.083, 0.056
Gag antigens 1 (CA)	12.139	0.034	0.924, 23.354	-0.013	0.653	-0.072, 0.046
Gag antigens 2 (MA)	15.722	0.037	0.940, 30.505	-0.007	0.810	-0.067, 0.053
Pol antigens 1 (RT p66)	-0.593	0.920	-12.365, 11.179	0.011	0.724	-0.053, 0.075
Pol antigens 2 (IN)	14.004	0.063	-0.759, 28.767	-0.006	0.860	-0.078, 0.065
Pol antigens 3 (RT p51)	0.322	0.979	-23.540, 24.185	-0.003	0.945	-0.088, 0.082

[1] Linear regression with robust standard errors, excluding measures after ART initiation

[2] Linear regression with robust standard errors, excluding measures after ART initiation, adjusted for measure at 12 months

Table 5. Participant Characteristics (n=100).

Variable	Frequency (%) or Mean (SD)					
	Uninfected (n=50)	**Any Infection (n=50)**	**Ascaris Infected (n=7)**	**Hookworm Infected (n=27)**	**Trichuris Infected (n=8)**	**Schisto Infected (n=16)**
Age	34.7 (9.7)	30.2 (9.4)*	26.6 (9.3)*	32.1 (10.0)	36.6 (10.0)	25.1 (5.0)*
Female	41 (82%)	41 (82%)	7 (100%)	24 (89.0%)	7 (87.5%)	11 (68.8%)
Helminth Infection						
Ascaris Only	0	3	3	0	0	0
Hookworm Only	0	19	0	19	0	0
Trichuris Only	0	5	0	0	5	0
Schistosomiasis Only	0	15	0	0	0	15
Mixed Infection	0	8	4	8	3	1
None	50	0	0	0	0	0
HIV status						
Log_{10} Viral Load	4.2 (1.0)	4.4 (0.8)	4.0 (1.2)	4.2 (0.9)	4.1 (0.7)	4.8 (0.5)*
CD4 Count (cells/mL^3) [1]	402.8 (162.9)	367.4 (250.4)	507.0 (440.7)	369.7 (289.0)	468.0 (475.3)	355.2 (234.4)
CD8 Count (cells/mL^3) [1,2]	751.6 (607.5)	871.8 (474.4)	958.7 (405.4)	895.3 (409.3)	884.2 (422.1)	772.6 (523.6)
Health Characteristics						
BMI (kg/m^2) [3]	23.0 (4.2)	21.4 (2.9)*	22.0 (4.1)	21.0 (3.0)*	21.3 (2.8)	21.5 (3.2)
White blood cells [4]	5.2 (4.8)	4.9 (1.7)	5.5 (1.4)	5.2 (2.0)	4.7 (1.5)	4.5 (1.1)
Red blood cells [4]	4.2 (0.8)	4.3 (0.7)	4.1 (0.4)	4.3 (0.5)	4.1 (0.6)	4.3 (1.0)
Hemoglobin [4]	12.0 (1.9)	11.7 (1.9)	12.0 (1.6)	11.5 (1.6)	10.5 (2.7)	12.3 (1.7)
Hematocrit [4]	35.8 (5.7)	35.3 (6.0)	35.6 (5.6)	34.9 (4.8)	32.4 (7.4)	37.2 (6.7)

[1] = median (interquartile range); [2] = 4 missing; [3] = 1 missing; [4] = 3 missing; * = statistically different compared to uninfected group ($p<0.05$)

Table 6. HIV antibody responses by helminth infection.

HIV-specific Antibody Responses *	Mean Difference	95% CI	P-value
Helminth Uninfected (n=50)	Reference	Reference	Reference
Any Helminth (n=50)			
HIV Component 1 (CA, vpu)	-0.158	(-1.05, 0.73)	0.726
HIV Component 2 (gp41, IN)	-0.226	(-1.03, 0.58)	0.579
Ascaris (n=7)			
HIV Component 1 (CA, vpu)	-0.022	(-1.13, 1.09)	0.969
HIV Component 2 (gp41, IN)	-0.797	(-2.18, 0.59)	0.253
Hookworm (n=27)			
HIV Component 1 (CA, vpu)	0.430	(-0.67, 1.53)	0.437
HIV Component 2 (gp41, IN)	-0.103	(-1.02, 0.81)	0.824
Trichuris (n=8)			
HIV Component 1 (CA, vpu)	0.728	(-0.88, 2.33)	0.368
HIV Component 2 (gp41, IN)	0.586	(-1.09, 2.26)	0.486
Schistosomiasis (n=16)			
HIV Component 1 (CA, vpu)	-1.506	(-2.44, -0.57)	0.002
HIV Component 2 (gp41, IN)	-0.972	(-1.87, -0.07)	0.035

* (predominant antibody responses in each PCA component)

Table 7. Characteristics of HIV-infected participants

	Frequency (%) or Mean (SD)			
	Study 1 Helminth infected vs. helminth uninfected		**Study 2 Dewormed vs. placebo treated**	
	Uninfected (N=50)	**Infected (N=50)**	**Placebo (N=19)**	**Albendazole (N=16)**
Clinical				
Female	41 (82%)	41 (82%)	17 (90%)	11 (69%)
Age at enrollment (years)	34.7 (9.7)	30.2 (9.4)	34.9 (9.0)	35.9 (10.7)
Log_{10} viral load	4.2 (1.0)	4.4 (0.8)	4.6 (0.7)	4.9 (1.1)
CD4 count (cells/mL^3)	430 (205)	441 (271)	507 (203)	476 (213)
Helminth Infection				
Ascaris	0	3	19	16
Trichuris	0	5	0	0
Hookworm	0	19	0	0
Schistosomiasis	0	15	0	0
Mixed	0	8	0	0
None	50	0	0	0
Helminth Risk Factors				
Primary school education	30 (60%)	28 (56%)	14 (74%)	12 (75%)
Unemployed	13 (26%)	13 (26%)	4 (21%)	7 (44%)
Farmer	10 (20%)	11 (22%)	9 (47%)	3 (19%)
# of children in the home	2.2 (1.6)	2.2 (1.6)	1.58 (1.26)	2.25 (1.61)
Piped water in home	4 (8%)	3 (6%)	1 (5%)	4 (25%)
Water source outside home	42 (84%)	40 (80%)	6 (32%)	7 (44%)
Environmental water source	4 (8%)	7 (14%)	12 (63%)	5 (31%)
Flush toilet in home	3 (6%)	3 (6%)	1 (5%)	2 (13%)
Pit latrine outside home	43 (86%)	47 (94%)	17 (90%)	13 (81%)

Table 8. Study 2 – Measles and tetanus antibody responses among dewormed compared to placebo treated, Ascaris and HIV co-infected individuals.

	Dewormed compared to placebo treated after 3 months		
Measles Response [1]	**Positive to Negative**	**No Change**	**Negative to Positive**
Placebo	3	16	0
Dewormed	0	15	1
P-value		0.15	
Log_{10} Tetanus Response [2]	**Coefficient**	**95% CI**	**P-value**
Ascaris Infected	-0.08	(-0.26, 0.11)	0.39

[1] Chi^2 test

[2] Linear regression with robust standard errors comparing dewormed to placebo at 3 months, adjusted for baseline

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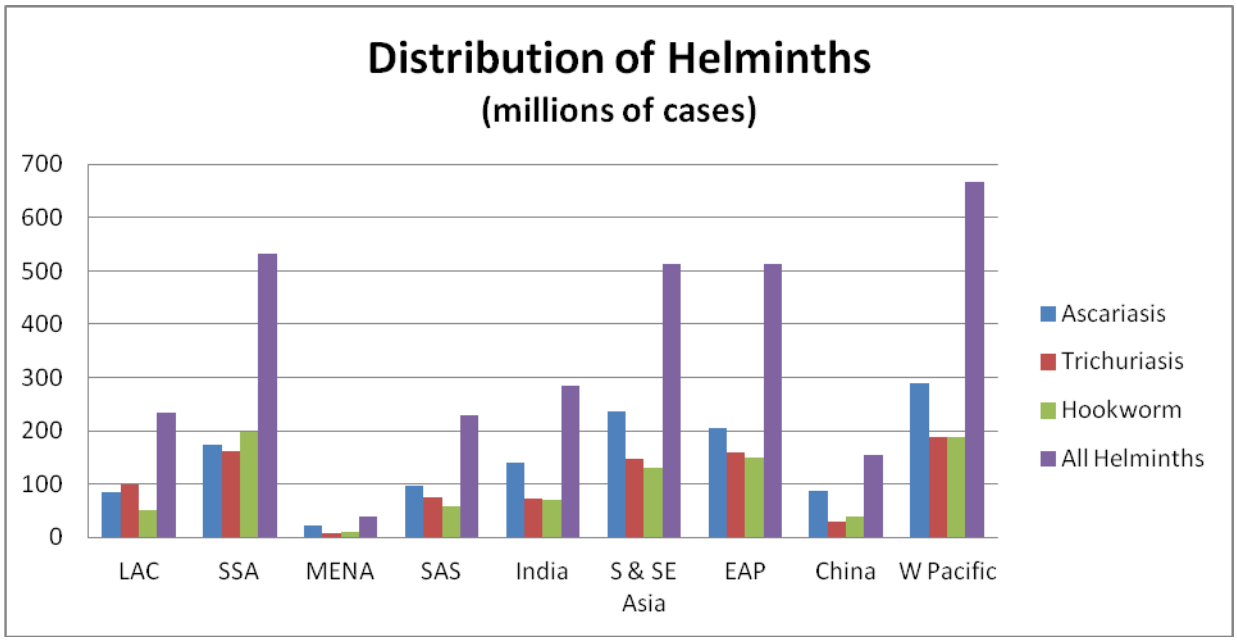

** adapted from: de Silva NR, et al. (2003) Trends Parasitol 19: 547-551 [18]

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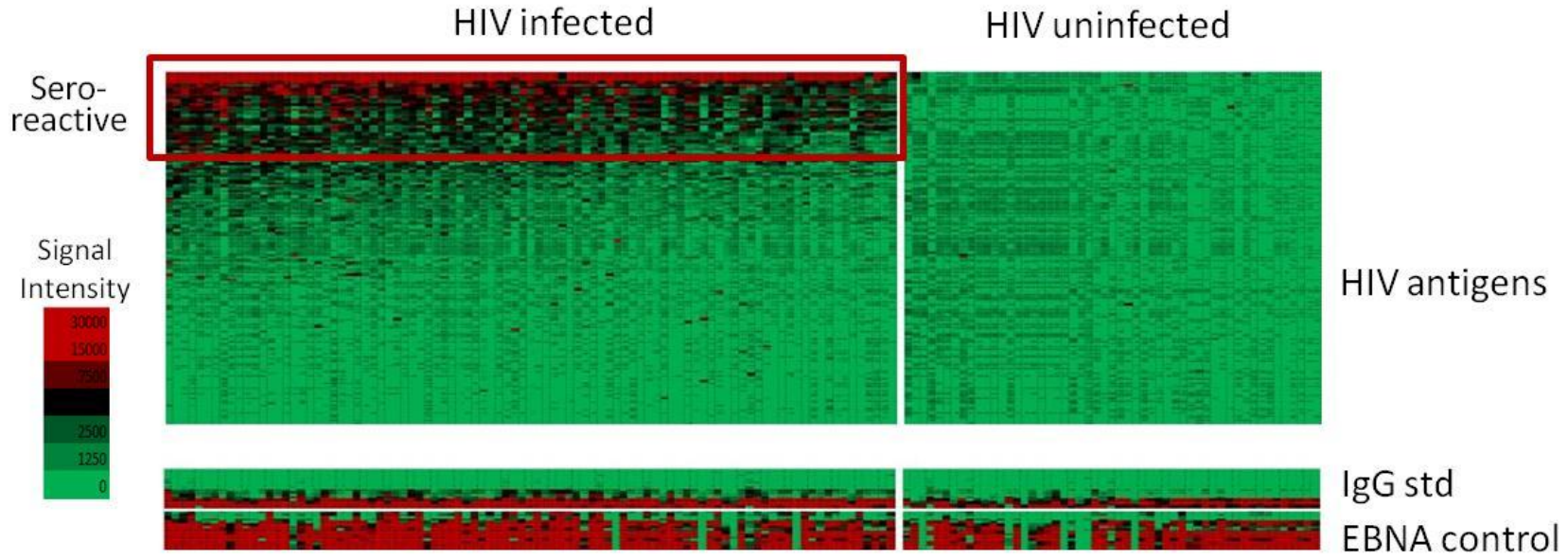

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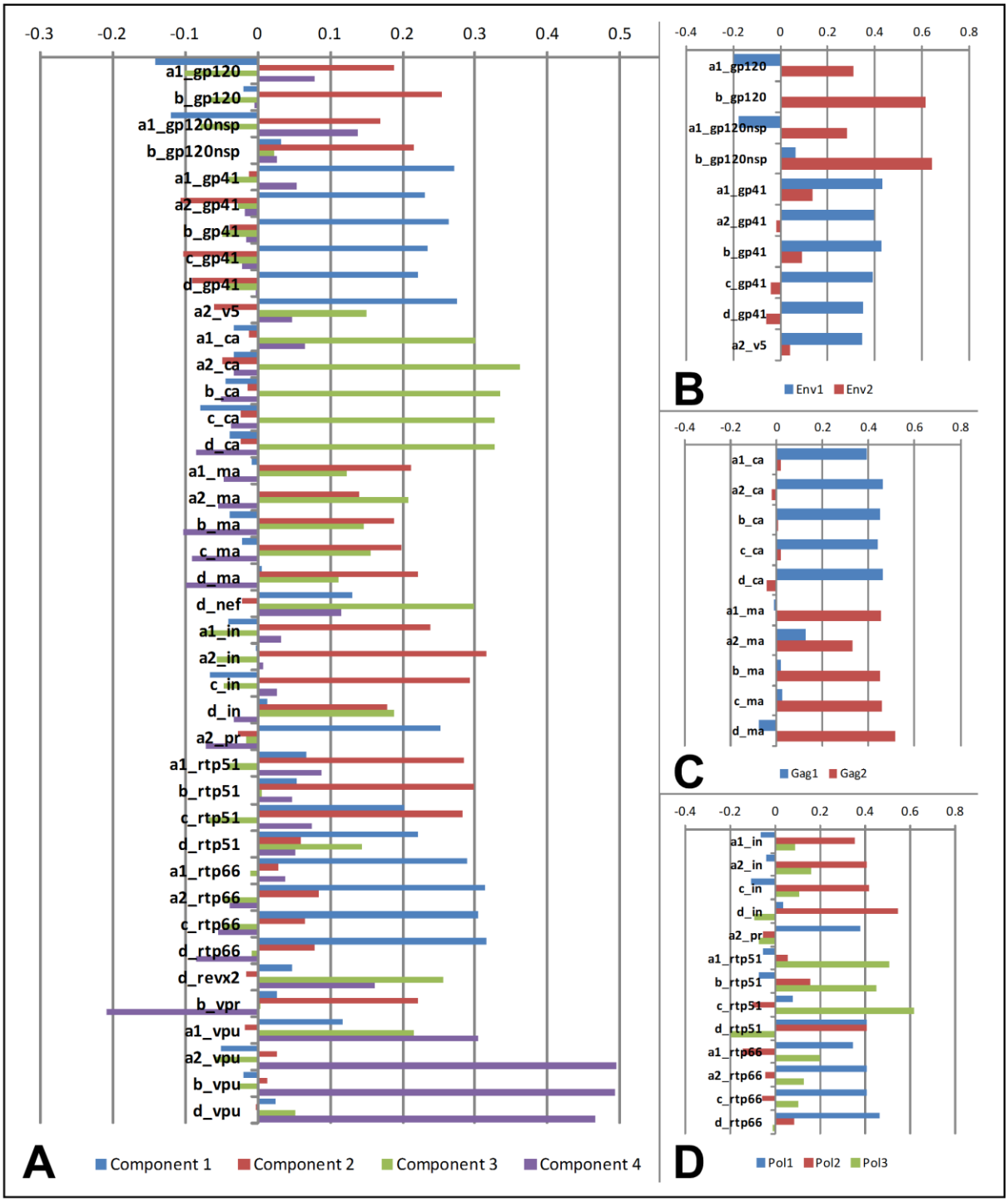

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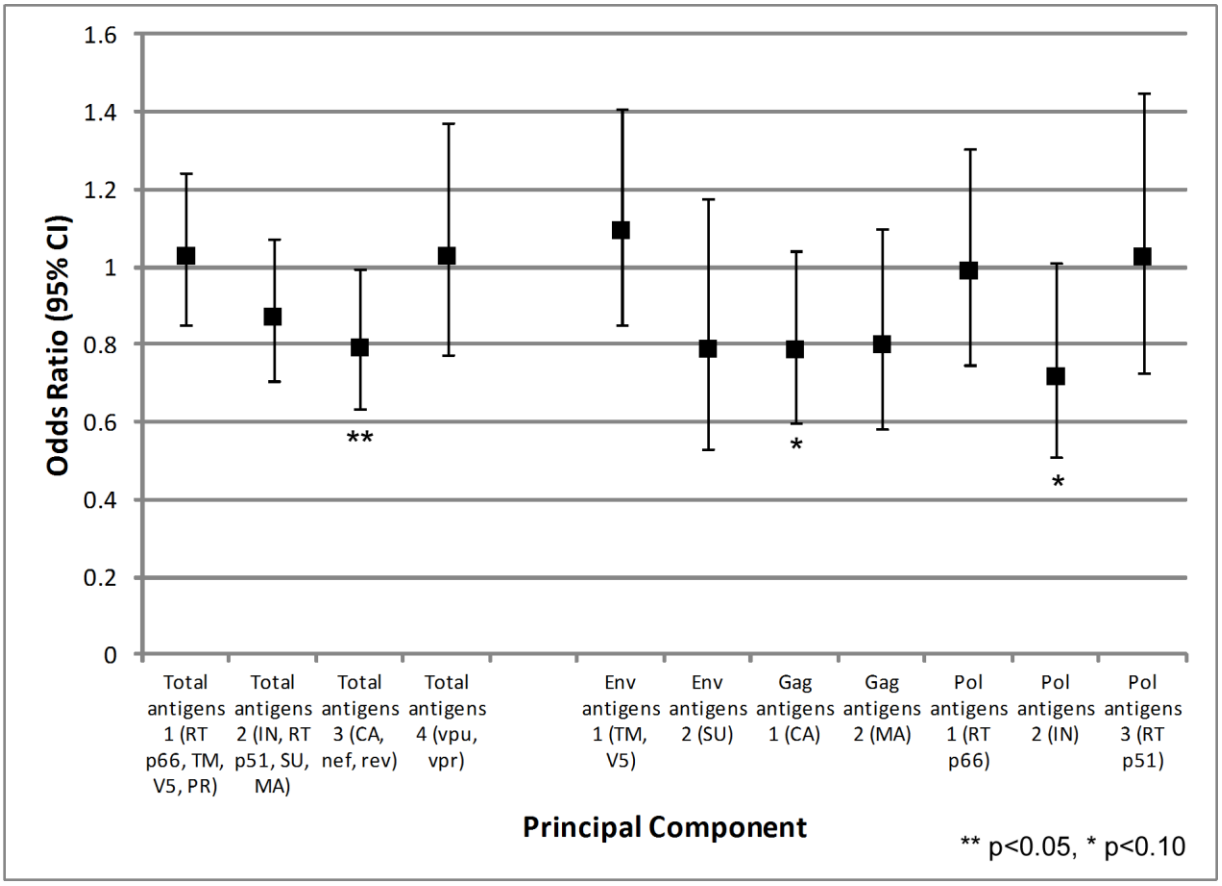

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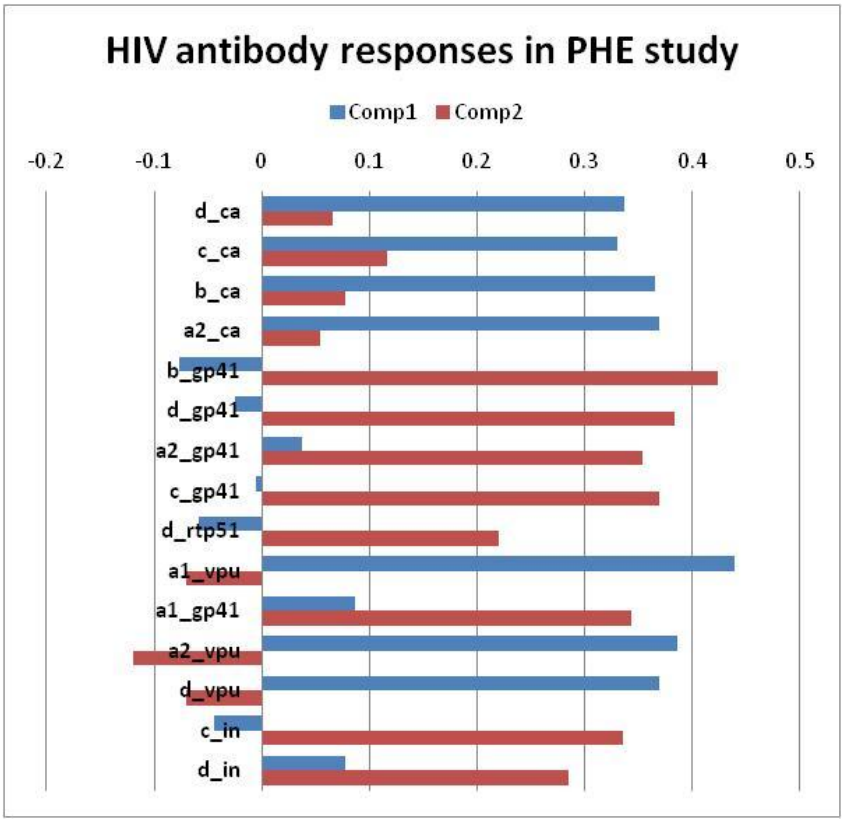

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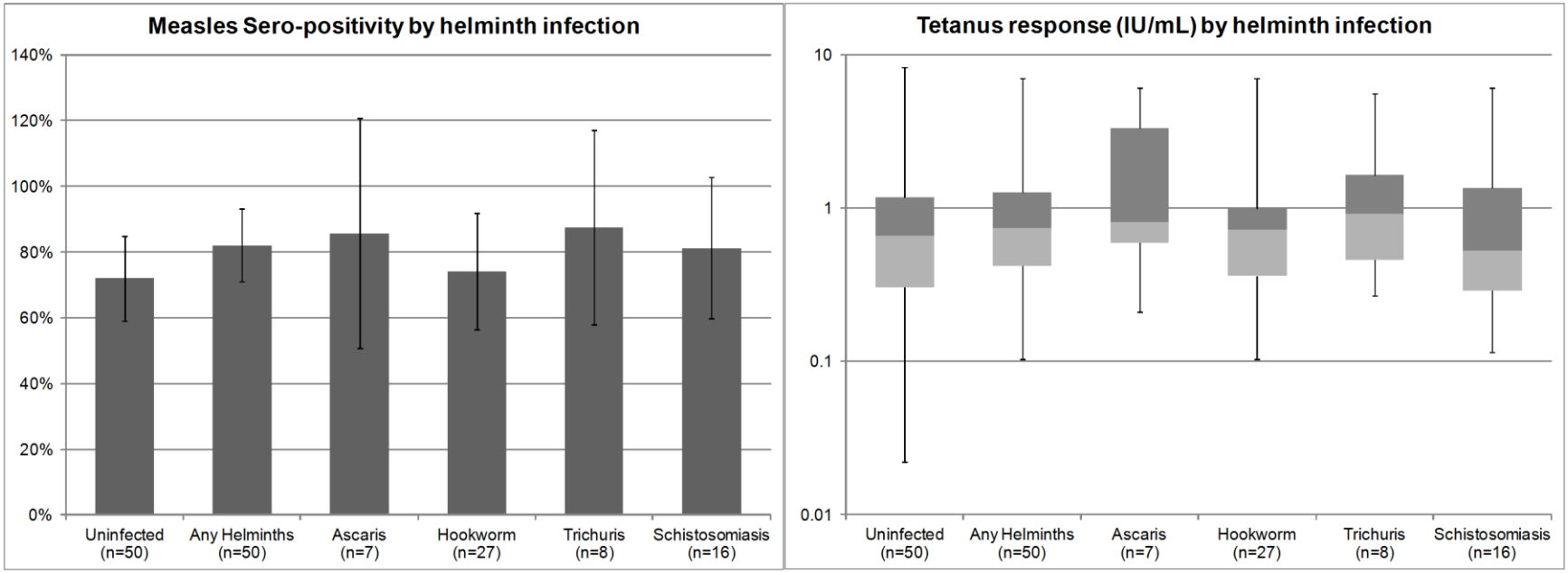

Note: mean sero-positivity with 95% confidence interval, and median tetanus response with box representing 25% and 75% percentile bounds, and whiskers representing minimum and maximum values.

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