Division of Infectious Diseases and Tropical Medicine

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Investigation of filarial associated immune modulation and effects on HIV susceptibility

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Abstract

Despite coordinated global activities supported by the WHO, lymphatic filariasis remains a large public health problem in many parts of the world. One important reason for this is the lack of treatment options available for the ~40 million people who suffer from chronic pathology (lymphedema or hydrocele), most of whom live in already poor communities in sub-Saharan Africa. Lymphatic filariasis is typically found in rural, hard to reach areas which can make it difficult for researchers to preserve samples from infected individuals. Because of this, we developed a novel whole blood flow cytometry method to minimize the amount of blood required (over the standard method), which maintains the integrity of extracellular markers, and eliminates the need for a -80°C freezer. This method was thoroughly tested and implemented in ongoing clinical trials in rural Ghanaian and Tanzanian field laboratories; our aim was to investigate immunological alterations in patients with lymphatic filariasis pathology as compared to those asymptomatically infected and control individuals, particularly regarding $CD4^+$ T cell activation and $CD8^+$ T cell exhaustion. We saw an increase of immune activation – defined by HLADR/CD38 expression on $CD4^+$ T cells – in lymphedema individuals as compared to filarial infected individuals without pathology as well as uninfected individuals from the same area. In addition, for the first time, we observed an increase of activation corresponding to lymphedema stage in samples from both Tanzania and Ghana. We next investigated the role of exhausted $CD8^+$ T cells in *W. bancrofti* infections by looking at a number of markers associated with exhaustion on both whole blood and PBMC samples since cells would be expected to proceed through activation to exhaustion with constant antigen exposure. Interestingly, we found that the cells from lymphedema patients displayed altered expression on exhausted $CD8^+$ T cell subsets when compared to uninfected controls and asymptomatic *W. bancrofti* infections. Also notable is that the asymptomatic *W. bancrofti*-infected individuals showed comparable results to the control samples in all of the markers examined. This thesis enhances the knowledge on the immune response of individuals to both asymptomatic *W. bancrofti* infections as well as the chronic pathology disease state through the development of a novel field method and flow cytometric analysis. This knowledge might help to prevent debilitating manifestations in filarial infected individuals in the future.

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Abbreviations

ALB	Albendazole
CFA	Circulating filarial antigen
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DEC	Diethylcarbamazine
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FC	Flow cytometry
FMO	Fluorescence minus one
FTS	Filariasis Test Strip
GAELF	Global Alliance to Eliminate Lymphatic Filariasis
GPELF	Global Programme to Eliminate Lymphatic Filariasis
HIV	Human Immunodeficiency virus
IL-10	Interleukin-10
IVM	Ivermectin
KLRG-1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte activation gene 3
LCMV	Lymphocytic choriomeningitis virus
LE	Lymphedema
LF	Lymphatic filariasis
MDA	Mass drug administration
MF	Microfilaria
MFI	Median fluorescence intensity
NTD	Neglected tropical disease
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-1	Programmed cell-death-1
TAS	Transmission assessment survey
Tim-3	T cell immunoglobulin and mucin-domain containing-3
WHO	World health organization

1. Introduction

1.1 Helminths

Helminth infections encompass a broad range of diseases caused by parasitic worms (helminths) – these range from nematodes (roundworms) causing lymphatic filariasis and onchocerciasis to platyhelminths (flatworms) causing schistosomiasis and cysticercosis (1). These helminths can be transmitted in a number of ways including the fecal-oral route, skin penetration through infected water or soil, or mosquito transmission (2). Helminths are so prevalent that they currently infect approximately 1/3 of the world's population (1, 3). Many helminth infections, such as schistosomiasis and lymphatic filariasis (LF), are classified as neglected tropical diseases (NTD) since they disproportionately affect poorer populations and are overshadowed by diseases such as malaria and tuberculosis (4, 5). Typically, NTDs do not cause death but rather lead to lifelong disabilities (e.g. blindness, elephantiasis, anemia and fatigue) or to a lower quality of life (e.g. missing school or work, social stigma).

1.1.1 Epidemiology and geographical distribution of lymphatic filariasis

LF is a helminth infection caused by either *Wuchereria bancrofti* (*W. bancrofti*), which is responsible for ~90% of LF infections, or one of the *Brugia* species (only in Asia) (6). LF affects approximately 65-68 million people residing in 73 countries worldwide (7-9). A majority of those infected maintain asymptomatic infections; for the rest (~40 million), the lymphatic system is impaired which leads to chronic pathology from hydrocele or lymphedema (LE), sometimes referred to as elephantiasis due to the large amount of swelling and change in the appearance of the skin (8, 10). Countries endemic for LF are primarily located in sub-Saharan Africa, south-east Asia, South America, and some islands in the Caribbean (Figure 1) (11). Since *W. bancrofti* is spread through a variety of mosquito species - including *Anopheles*, *Culex, Aedes,* and *Mansonia* depending on the region - areas which are endemic for LF tend to be tropical or subtropical (11).

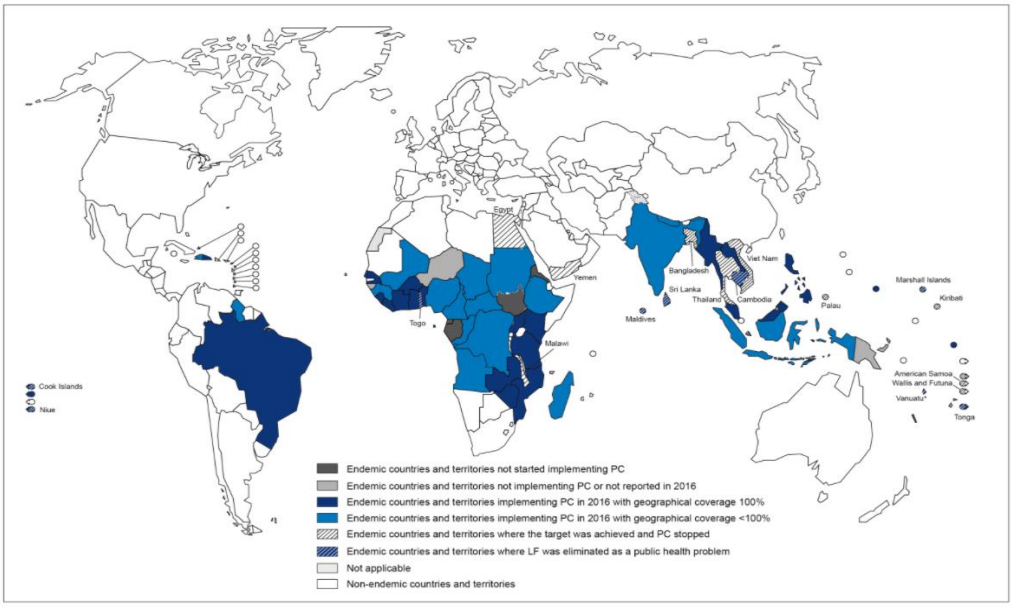


Figure 1: Geographic distribution of lymphatic filariasis.
Source: World Health Organization 2020

1.1.2 *W. bancrofti* life cycle

While *W. bancrofti* can be transmitted by a variety of mosquitos, the life cycle remains the same throughout all of them. LF is a two host disease; *W. bancrofti* proceeds through half of its life cycle in one of the above-mentioned mosquito hosts and is then transmitted to a human host through the mosquito blood meal (Figure 2). The life cycle involves several stages which begins with microfilariae (MF) entering the mosquito during a blood meal, losing the sheath in the mosquito's stomach, and migrating through the midgut to the thoracic muscles where it begins to develop into the first-stage larvae. Over time it continues to grow and molts twice before it develops into the infective L3 larvae stage (18-23 µm diameter and 1.5 mm long); at this point the larvae make their way to the mosquito's head and proboscis where it is able to be transmitted to the human host when the mosquito feeds. The L3 larvae migrates to the lymphatics of the human host where they can molt and develop into adult worms over a 5-18 month period. These adult worms can live and reproduce the sheathed MF (7.5-10 µm wide and 244-296 µm long) anywhere from 5-10 years. These MF typically live 6-24 months and will move throughout the lymphatic vessels and bloodstream over that time (12). The MF are released into the bloodstream with nocturnal periodicity, where they can be taken up by the mosquito again during a blood meal to continue the life cycle as before (13).

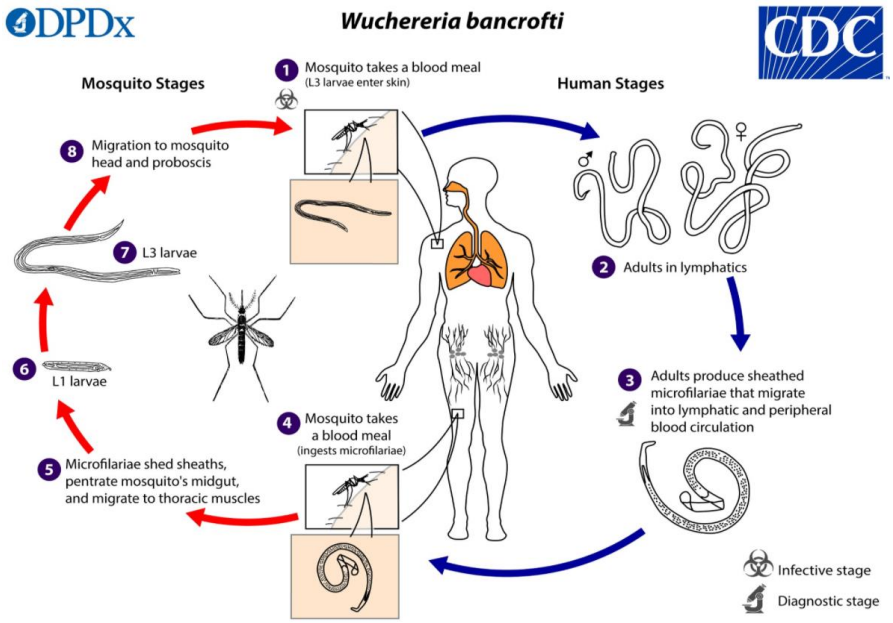


Figure 2: Life cycle of Wuchereria bancrofti.
Source: CDC 2020

1.1.3 International goals of MDA treatment

While there is no approved medication for those already suffering from the chronic pathology of LE or hydrocele, there are mass drug administration (MDA) programs in place in many countries to reduce the spread of LF. According to the Global Alliance to Eliminate Lymphatic Filariasis (GAELF), the purpose of MDA treatment is to treat entire areas with combinations of Ivermectin (IVM) plus Albendazole (ALB) and/or Diethylcarbamazine (DEC) in order to interrupt MF transmission to a point where it is no longer a threat for mosquitos to uptake the MF and further transmit them to other individuals (9, 15, 16). The World Health Organization (WHO) recommends that public health campaigns treat endemic areas for 5-7 years in order to achieve treatment for at least 65% of the population (17). The rates of transmission are assessed with transmission assessment surveys (TAS) which periodically assess and evaluate when this threshold has been reached in endemic areas. This is done with a combination of mapping activities, MDA treatment, and post MDA surveillance (Figure 3).

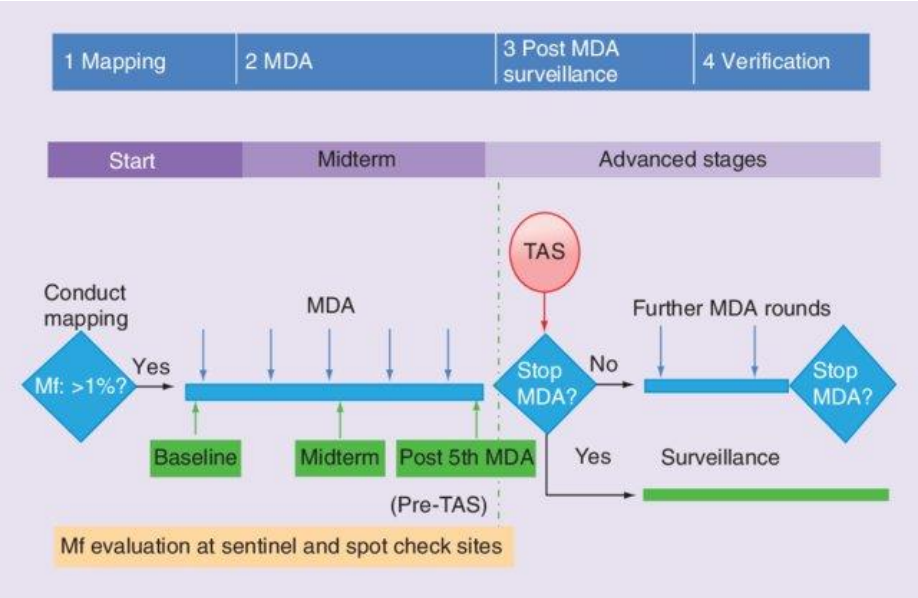


Figure 3: Timeline for mass drug administration treatment programs.
Source: Rebollo et al. *2013. Reproduced with permission from RightsLink®/Elsevier, obtained on 09 June 2021.*

A worldwide task force called the Global Programme to Eliminate Lymphatic Filariasis (GPELF) was created by the WHO in 2000 whose purpose is to use systematic MDA treatment in order to disrupt transmission of LF and ease the suffering of those who are already affected (16). Figure 4 shows how widespread treatment in endemic areas has already greatly reduced the burden of disease from 2000-2018 in sub-Saharan Africa (18). As of 2019, there were only 3 endemic countries who have not started MDA programs at all and 16 countries who have been able to declare they are no longer endemic for LF due to the MDA programs, as is seen in Figure 5 (9). There still remain about 893 million people in 49 countries who are at risk of becoming infected with *W. bancrofti* (6).

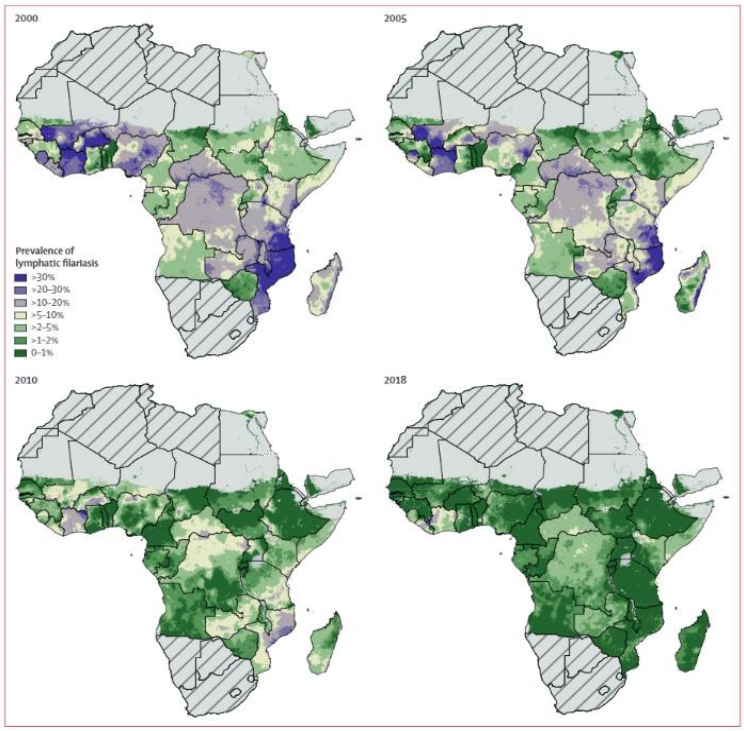


Figure 4: Endemic regions for lymphatic filariasis assessed before and after start of MDA programs from 2000-2018.
Source: Local Burden of Disease 2019 Neglected Tropical Diseases Collaborators. Reproduced with permission from RightsLink®/Elsevier, obtained on 09 June 2021.

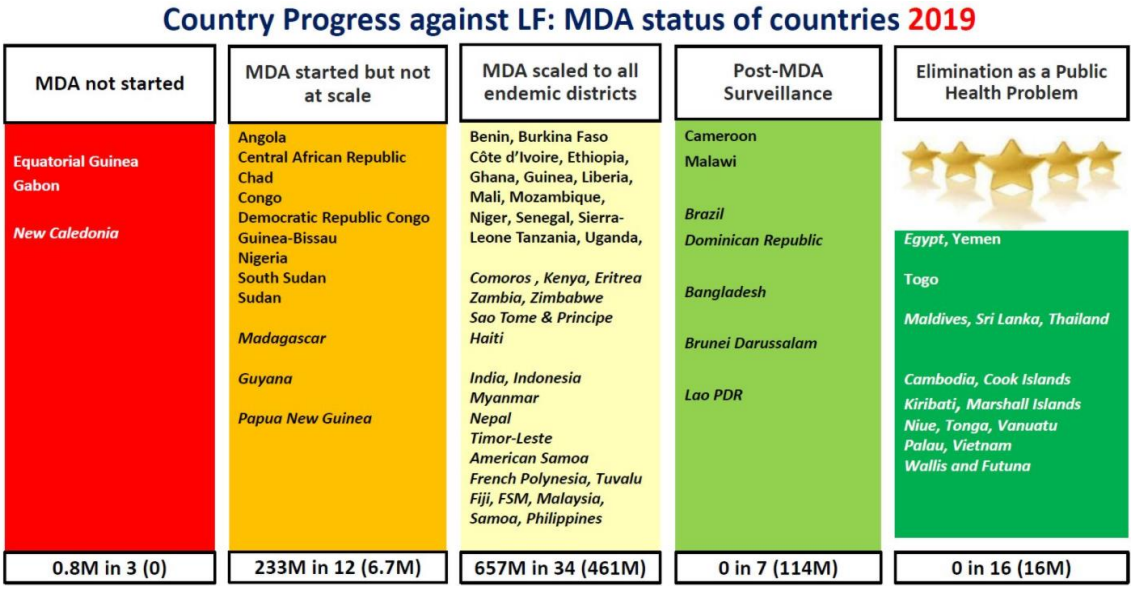


Figure 5: MDA status for endemic countries against lymphatic filariasis (2019).
Source: WHO 2019

1.1.4 Treatment of *W. bancrofti*

The main drugs used in the MDA programs mentioned above are combinations of IVM plus ALB and/or DEC which are effective against the *W. bancrofti* MF

rather than the adult worms. IVM works to paralyze the MF by blocking the glutamate-gated chloride channels (19). Once this happens, the MF are moved to regional lymph nodes where they are quickly killed through effector cells. In addition, it is suspected IVM also prevents reproduction by the adult females by paralyzing the pharynx which deprives the worm of iron, thus inhibiting growth and reproduction (19). The role that ALB plays is not as clear as that of IVM; it may not have a direct effect on the MF, but it seems to play an important role in different gastrointestinal helminths which improves the overall situation (20). DEC is a drug known to be effective in other human filarial infections (e.g. hookworm, ascariasis) and can affect the parasites at many different stages during the life cycle, however the mechanism by which it works in LF is not yet known (19, 21). In areas co-endemic for onchocerciasis, DEC is not used since it can cause severe side effects (22). Combinations of these 3 drugs has thus far proven effective in killing the MF but not in killing the worm, which is why ongoing MDA treatment is needed over many years (19, 23).

One macrofilaricidal drug that has been tested for a number of years is Doxycycline, which acts rather on the endosymbiont *Wolbachia*, crucial for the survival of many filarial worms, including *W. bancrofti* (24, 25). This therapy requires treatment for only ~6 weeks with the drug as an alternative to the 5-7 years of MDA in the above-mentioned therapies. Additionally, Doxycycline has been shown to reduce the stage of mild to moderate LE cases, regardless of if they have an ongoing infection (26). While results with Doxycycline have been promising, the drug is not recommended in children or pregnant women, limiting its universal use.

1.1.5 Testing for *W. bancrofti*

There are a number of ways to test a person when filarial LF is suspected: Directly observing MF in the blood (Giemsa blood smear, membrane filtration), antigen testing (Filariasis Test Strip, TropBio Og4C3 ELISA), or by using polymerase chain reaction (PCR) to test for filarial deoxyribonucleic acid (DNA) (27-29).

The Giemsa blood smear is a versatile method that has been employed for the identification of many malaria and blood parasites (30) and has been long used as a field method for identifying LF MF (31). With the use of Giemsa stain blood

smears, it is possible to differentiate between *W. bancrofti* and other filarial infections which could also be endemic in the area (e.g. *Mansonella* species). The Giemsa blood smear can also be paired with membrane filtration in order to determine the concentration of filaria in a blood sample (31). However, these methods can be difficult to perform due to the nocturnal periodicity of the MF, meaning they are only active and visible when night-blood is taken (29). The method can also miss individuals who have low numbers of MF which could lead to underdiagnoses of these areas (28).

Antigen tests work through detection of the circulating filarial antigen (CFA) that is released from the adult worm into the blood of the human host, meaning the blood to be used can be taken at any time (not subject to nocturnal periodicity) (27). Weil and colleagues developed a successful antigen test in 1984 which was more sensitive than the blood smears, but not practical to use for public health purposes (32). The first new test that was used for public health campaigns was the ICT Filariasis Test; it was first developed in 1997 before being bought by Binax, Inc. (Portland, ME) and becoming the BinaxNOW® Filariasis card test in 2000 (33). This test was widely used in MDA programs throughout the world. Commonly used antigen tests today include the Alere Filariasis Test Strip (FTS) (previously BinaxNOW® Filariasis card test) or the enzyme-linked immunosorbent assay (ELISA) TropBio Og4C3 test (referred to hereafter as TropBio) (Figure 6).

Antigen tests have become widespread due to their ease of use, non-invasive techniques, availability, and the higher sensitivity levels as compared to microscope MF detection (28, 34). The FTS test has even been promoted by the WHO to be used as an indicator for when to stop widespread MDA treatment in areas as part of the GPELF (35). The TropBio is not as quick or easy to use so it cannot be used as a field method; however, quantitative results can be obtained (36).

Figure 6: Use of the Alere Filariasis Test Strip in the field and the TropBio ELISA assay in the laboratory.
Source: Inge Kroidl. Pictures were obtained from the LEDoxy trial (ISRCTN14042737) within the TAKeOFF project funded by Federal Ministry of Education and Research (BMBF) and Health Africa project.

1.1.6 Morbidity management

As previously mentioned, about 2/3 of people infected with LF remain asymptomatic, with the rest developing disfiguring hydrocele or LE. The Dreyer scale (37) is commonly used to classify the progression of LE cases. Patients are examined and given a number (1-7 with 1 being the lowest) based on qualification criteria including appearance of skin, amount of swelling, and presence of mossy lesions (Figure 7). In stage 1, the swelling of the limb is reversible overnight and is only present during the day. Stage 2 includes swelling that is present throughout the day and night. Stage 3 progresses to include shallow skin folds and the patients are more prone to have acute attacks. In stage 4, patients also have knobs on the skin. Stage 5 includes deep skin folds in the affected limbs and they experience acute attacks more often. In stage 6, patients experience mossy lesions and have many entry lesions. Stage 7 is the highest stage and occurs when the patient is no longer able to take care of themselves and perform any daily activities.

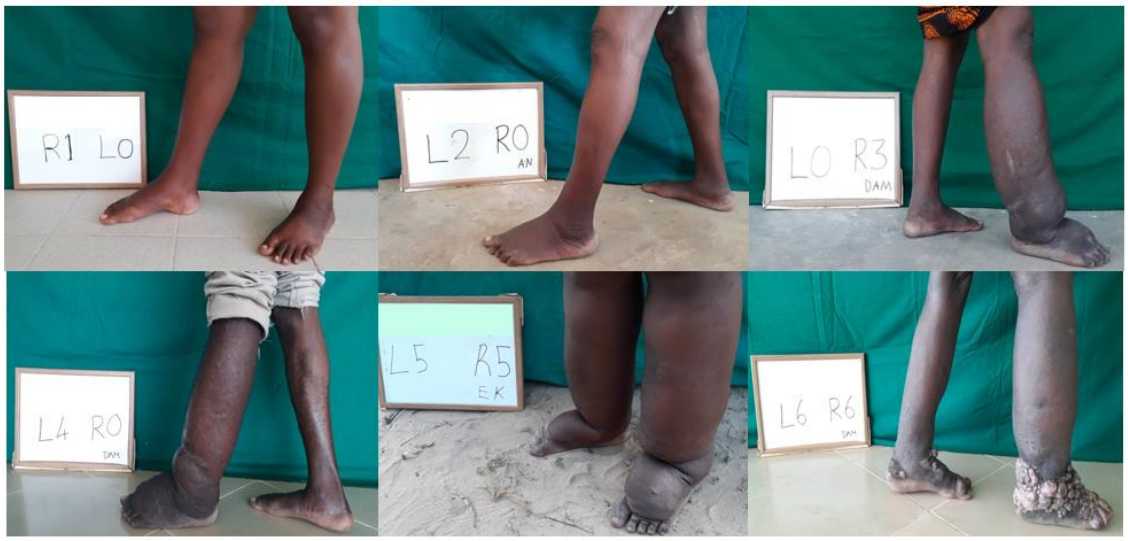


Figure 7: Representative leg lymphedema stages 1-6 based on the Dreyer scale for lymphedema classification.
Source: Leonard Masagati. Pictures were obtained from the LEDoxy trial (ISRCTN14042737) within the TAKeOFF project funded by Federal Ministry of Education and Research (BMBF) and Health Africa project.

One of the reasons LF remains such a large public health burden today, despite the fact that there are large MDA campaigns to reduce transmission and spread, is due to the fact that there is no widespread medication to help individuals once they have LE (apart from preliminary data on Doxycycline). Campaigns teach individuals correct hygiene practices and remind them to wear shoes to help with infections. These activities can help relieve suffering but do not result in long term reduction of LE pathology. Those suffering can also experience acute dermatolymphangioadenitis attacks (ADLA), caused by secondary fungal and bacterial infections that enter through lesions in the skin (associated more often with higher stages of LE). ADLA can then lead to accelerated progression of LE development (38-40). Antibiotics can be used to help treat the bacterial infections which in turn reduces the inflammation (41).

1.1.7 Social stigma

Not only do these individuals suffer from potentially debilitating pathology, but it can also lead to social stigma and discrimination, mental health problems, difficulties performing daily tasks, and higher rates of poverty (6, 42, 43). Quality of life is greatly affected by the amount of stigmatization these individuals feel which

varies based on the visibility of the disease (44). Women in particular suffer great difficulties when searching for a spouse, leading to extra burdens on their families along with fear and anxiety for the women themselves (45, 46).

1.2 Immune response to pathogens

The immune system provides protection against any foreign invaders, including viruses, bacteria, foreign tissues or cells, or parasites (such as helminths). In order to achieve this, a complex interplay between all components of the immune system is required to discern between the body's own "self" cells and tissues from other harmful "non-self" products (47, 48). The initial response comes from the innate immune system and is later supported by the adaptive immune system; these two systems work together via signaling molecules (e.g. cytokines) to provide protection (49).

1.2.1 Immune evasion

Helminths and humans have been coevolving over millions of years in order to find ways to exist together; by relying on a variety of immune evasion tactics (e.g. downregulating the host's immune system), helminths are able to prevent expulsion from the host before reproduction and proliferation (50). Chronic helminth infections result in immune responses that often allow for a continuation of infection rather than parasite expulsion which could cause sever pathology for the host. The downregulation of the host's immune system can have a variety of other consequences, including lower levels of autoimmune diseases or reduced allergies due to the host having a diminished ability to develop "over-active" immunity (Figure 8) (51-54).

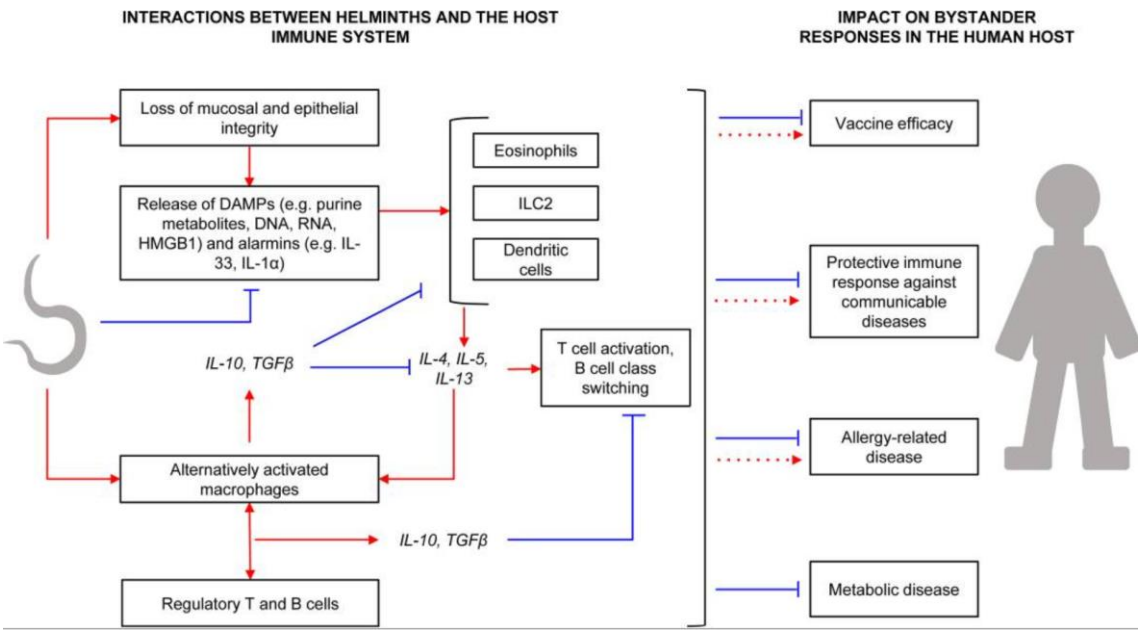


Figure 8: Interactions between the helminth and the immune system of the host can have a variety of other effects.
Source: Sanya et al *2017. Reproduced with permission from RightsLink®/Elsevier, obtained on 11 June 2021.*

1.2.2 Immune response to *W. bancrofti*

Individuals infected with *W. bancrofti* can display a variety of infection statuses and clinical manifestations, including infected MF-negative, infected MF-positive (both asymptomatic), or chronic pathology (hydrocele or LE). Many uninfected individuals (endemic normal) also reside in these filarial endemic areas where they are constantly exposed to *W. bancrofti* but do not develop any of the above mentioned infection statuses. It has been suggested that those asymptomatic individuals have a distinct immunoregulatory environment which allows for the persistence of the MF within the host through downregulation of the immune response (50, 55, 56). The typical immune response of asymptomatic individuals is characterized by increased expression of regulatory molecules such as interleukin-10 (IL-10), TGF-β, and cytotoxic T Lymphocyte antigen 4 (CTLA-4), along with the increase in regulatory T and B cells, dampening of the Th1 immune response (e.g. inflammatory cytokine IFNγ), and an induction of the Th2 response (e.g. IL-4, IL-5, IL-9, IL-10, IL-13) (50, 57-62). T cells are crucial for eliminating filarial infections; studies in mice have shown that those lacking T cells are vulnerable to infections with the *Brugia* parasites (63, 64). The combination of all of the immune responses ensure MF can survive long-term in the human host as

well as preventing the onset of chronic pathology. Individuals who suffer from chronic pathology, often after the parasite is no longer present within the body, display different immunological patterns (Figure 9). They tend to have suppressed expression of regulatory T cells (Tregs), elevated numbers of cells typical in the Th1 and Th17 response, constant immune activation, and increased pro-inflammatory responses (50, 61, 62, 65).

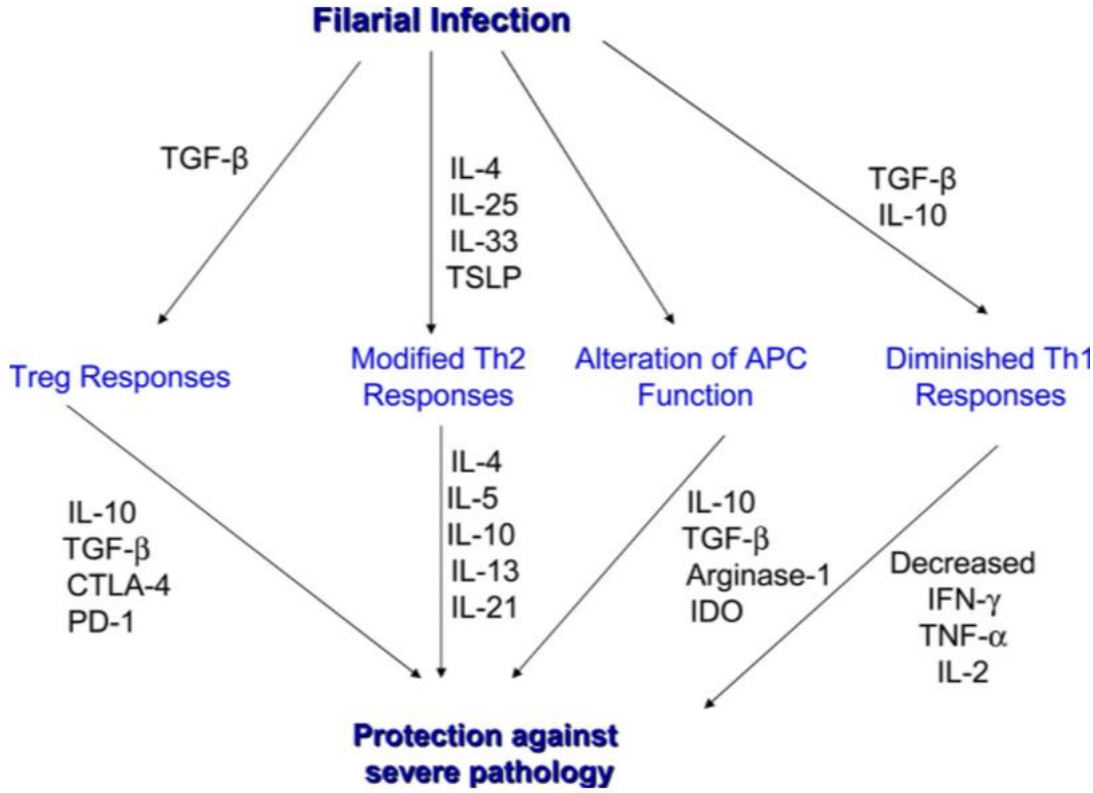


Figure 9: Characteristic immune responses seen in filarial infections.
Source: Babu and Nutman 2012. Reproduced with permission from RightsLink®/Elsevier, obtained on 11 June 2021.

1.2.3 CD4$^+$ T cell activation

Large areas of the world which are endemic for LF have also been largely affected by the human immunodeficiency virus (HIV) epidemic (66), particularly in sub-Saharan Africa. While there are a number of factors contributing to this overlap, one suggested hypothesis is that infections, in particular helminth infections, lead to increased systemic immune activation in infected individuals. These helminth infections in turn lead to an increased Th2 response, leading to an overall increase in susceptibility to HIV infection (67-70). Chronic immune activation,

along with immunodeficiency, is a prominent feature in the progression of HIV-1 disease; it has been shown since 1990 that an increase in the expression of various activation markers is associated with disease progression (71-73). These activated $CD4^+$ T cells are often important for HIV replication and can even be reservoirs for HIV. It has also been shown that those individuals who display lower levels of immune activation are less susceptible to HIV infection (14, 74-77).

Also reported is that individuals with other helminth infections - *Schistosoma mansoni*, *Trichuris trichiura*, or *Ascaris lumbricoides* – display higher levels of activated $CD8^+$ and $CD4^+$ T cells than those uninfected with any of the helminths that were tested for (78). While not tested for in the aforementioned study, *W. bancrofti* infection was later found to lead to an increased susceptibility to HIV, possibly due to the higher levels of immune activation in the filarial infected individuals that helped disseminate the virus early on (79). Indeed, cross-sectional studies have shown that the prevalence of HIV positive individuals was greater in those infected with *W. bancrofti* as compared to non-filarial infected individuals (80).

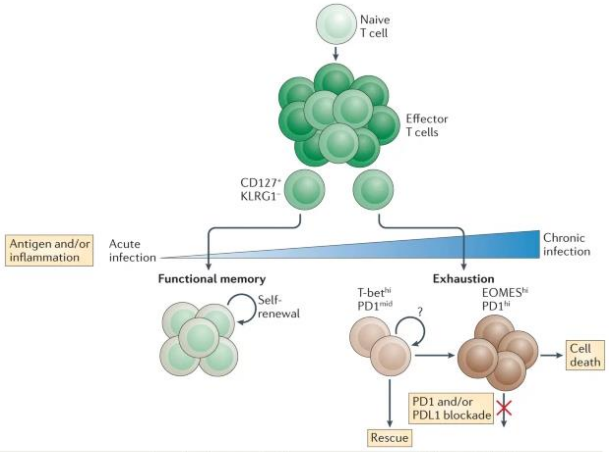
HLADR, a MHC class II antigen presenting cell, and CD38, a transmembrane glycoprotein, are used either alone or in combination to characterize activation on $CD4^+$ and $CD8^+$ T cells in helminth infections (78). Cells expressing HLADR alone are found within memory populations, while cells expressing CD38 alone are found within the naïve populations in both $CD4^+$ and $CD8^+$ T cells. However, $CD4^+$ or $CD8^+$ T cells expressing the combination of $HLADR^+/CD38^+$ were almost entirely found on the memory populations. In both helminth infections in general and *W. bancrofti* specific infections, studies have reported increased levels of $HLADR^+/CD38^+$ $CD4^+$ T cells among helminth infected individuals (78, 79).

1.2.4 $CD8^+$ T cell exhaustion

In order to sufficiently control helminth infections, there needs to be a cooperation between various cell types, including B and T cell populations (81-83). In particular, $CD8^+$ T cells are very important in recognizing and subsequently eliminating various intracellular pathogens. $CD8^+$ T cells have been well-studied in other chronic diseases (e.g. HIV, hepatitis C, cancer) but there is a lack of information

available on the role of $CD8^+$ T cells in filarial infections, specifically *W. bancrofti* (84-86). One such cell type which is hypothesized to play a role in filarial infections is the exhausted $CD8^+$ T cell subset.

Exhausted $CD8^+$ T cells are a distinct subset of cells from the effector and memory $CD8^+$ T cells, as can be seen in Figure 10. This subset of cells occurs when there is persistent and continuous antigen exposure and chronic inflammation which causes the cells to be in a state of constant activation, leading to exhaustion. Wherry has very succinctly defined the term T cell exhaustion as a state of differentiation with several typical features including step-wise losing effector functions, inhibitory receptors being simultaneously co-expressed, changed expression of transcription factors, and the failure of cells to proceed to quiescence (84-87). This hierarchical process typically begins with the loss of IL-2 production, followed by TNF-α, and in the late stages of exhaustion, IFN-γ (83, 85, 88). The late stages of exhaustion (after loss of TNF-α and IFN-γ) are typically associated with failures in controlling pathogens (85, 88).



Feature	Functional memory T cell	Exhausted T cell
Proliferative potential	+++	+/−
Cytokine production	+++	+/−
Memory markers (e.g. CD44, CD62L, CD127 or CXCR3)	+++	+/−
Inhibitory receptors (e.g. PD1, LAG3, CD160 or 2B4)	−	+++
IL-7- and/or IL-15-driven self-renewal	++	−
Antigen dependency	−	+

Figure 10: Exhausted T cell progression.
Source: Wherry and Kurachi 2015. Reproduced with permission from RightsLink®/Elsevier, obtained on 09 June 2021.

Studies performed in mouse models show the importance of two transcription factors - T-box transcription factor (Tbet) and Eomesodermin (Eomes) - for

memory formation (89-92). Tbet is important in the regulation of effector functions and has been shown to be downregulated in $CD8^+$ T cells during chronic infections; this downregulation is then associated with T cell dysfunction (93). On the other hand, Eomes plays a role in regulating proteins within the memory $CD8^+$ T cell populations and was shown to be upregulated during chronic lymphocytic choriomeningitis virus (LCMV) infections within the exhausted $CD8^+$ T cells (93). While these transcription factors have different functional roles, the ratio between these two has been used to further examine the exhausted $CD8^+$ T cell subset (89).

Along with Tbet and Eomes, a number of phenotypic markers have also been used to characterize the $CD8^+$ T cell exhausted populations; these include programmed cell-death 1 (PD-1), lymphocyte activation gene 3 (LAG-3), killer cell lectin-like receptor subfamily G member 1 (KLRG-1), CD39, T cell immunoglobulin and mucin-domain containing-3 (Tim-3), and IL-10. Many have been shown to be upregulated in response to chronic viral infections, as well as having helminth infection associated immune responses (83, 94-96).

1.3 Field work in resource-limited settings

While field tests for filariasis can be challenging, so too can many laboratory procedures in resource-limited settings. These laboratories often lack the required specialized equipment (e.g. freezers, centrifuges, safety cabinets, Laminar flow), and if the equipment is present, it can undergo quicker wear and tear due to varying weather conditions (e.g. humidity), as well as frequent power supply shortages (97, 98). A typical procedure in a field setting is to collect peripheral blood mononuclear cells (PBMC) which are then transported to a more equipped lab for later flow cytometry (FC) processing (100-103). However, it is a far from ideal situation; there can be power cuts during the long centrifugation step, limited storage options available after the PBMCs are isolated, and the procedure itself is rather expensive and time-consuming (97, 103). One additional factor to take into consideration is the large blood draw required to isolate a sufficient number of PBMCs, making this procedure difficult to be performed on infants and pregnant

women (104-106). Furthermore, participants are not always willing to provide such large blood draws due to cultural beliefs (104).

Another option is to employ the use of whole blood procedures, which utilize smaller amounts of blood than PBMCs. These methods can work well for some markers (e.g. CD3, CD4); however they also use lysing and fixation steps which include paraformaldehyde. This can potentially change the epitope of some cell markers and become problematic for later extracellular staining (107-109). Previous studies have tested multiple whole blood fixation and cryopreservation methods in order to identify T cell, B cell, and natural killer cell subsets but have stopped short of identifying markers relevant for many research activities (110).

1.4 Objective

The aim of the work presented here is to better characterize and add to the knowledge of what is known about activation and exhaustion within *W. bancrofti* infected individuals along with those suffering from chronic LE pathology.

This was done by developing a novel flow cytometry field method to solve the problem of using flow cytometry for cell characterization in resource-poor settings. This method was then used in ongoing studies and clinical trials to examine a number of markers associated with activation and exhaustion within individuals presenting leg lymphedema, *W. bancrofti*-infected individuals, and endemic normal control individuals. The findings presented add to the general knowledge surrounding LF which can help guide the medical professionals providing advice and treatment for this infection.

2. Material and Methods

2.1 Study design and protocol testing

The whole blood method presented was tested using healthy control volunteers from participants residing in Munich, Germany. Both genders were sampled and volunteers came from different ethnic backgrounds.

For the field study data, samples were taken from two regions as part of an ongoing TAKeOFF-LEDoxy clinical trial (111): The Upper East Region of Ghana and the Lindi Region of Tanzania. Additional samples were collected in Ghana during screening and recruitment for a DFG funded project (RHINO). These studies have gained ethical approval from the National Institute for Medical Research (NIMR) in Dar es Salaam, Tanzania (NIMR/HQ/R.8a/Vol.IX/2693), Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana (CHRPE/AP/144/20, GHS-ERC-007/97/2017), the Division of Infectious Diseases and Tropical Medicine at Ludwig-Maximilians-Universität in Munich, Germany (18-377, 17-858), and the Institute of Medical Microbiology, Immunology, and Parasitology (IMMIP) at the University Hospital Bonn, Germany (Lfd. Nr. 041/18, 359/17). Prior to participating in the study, all individuals provided written informed consent.

2.1.1 Study population and parasitic assessments

For the LE participants in the LEDoxy study, leg stage was defined and classified according to the Dreyer staging protocol (37) from stage 1-7, as described above. In the next data presented, we also recruited participants who were classified as either endemic normal (EN) or *W. bancrofti*-infected (Wb-inf.) in addition to the LE participants in order to further examine the markers of activation and exhaustion. The EN and Wb-infected cohorts were tested for the presence of CFA; first in the field with the FTS (previously Alere, now Abbott Laboratories, Chicago, USA) and later using stored samples with the TropBio ELISA (Cellabs, Brookvale, Australia). The EN group was defined as being negative for both the tests, had

no indications of LE pathology, and had been living in the study area for a minimum of 5 years. The Wb-infected group was positive for both of the tests but also had no indications of LE pathology. Appendix A shows the descriptive statistics for each of these cohorts.

2.2 Whole blood flow cytometry panel design

The design of the flow cytometry panel used in all whole blood methods presented here included a number of initial steps: First, titration testing was done to find the appropriate concentrations of all antibodies, next compensation controls were used in order to control for spectral overlap, and finally fluorescence minus one (FMO) controls were performed to ensure accurate gating. After these initial steps were completed, 2 separate FC panels were developed which included 7 and 9 extra- and intracellular antibodies, respectively. Appendix B shows all antibodies, clones, and companies which were used in the whole blood method.

2.2.1 Whole blood method field sample processing and extracellular staining

For the whole blood method, sample processing began with collection of 10 mL of venous blood in sodium heparin tubes (Sarstedt, Nümbrecht, Germany). Blood was processed immediately during establishment of the method. However, in the field laboratories in Ghana and Tanzania, blood was collected in a remote location before storage and transportation in cool boxes to the field laboratory. Blood was then processed within 8 hours of sample collection time.

100 µL of whole blood (per FC panel) was put into a separate tube for processing. Next, the extracellular antibodies were added to each tube before incubating for 30 minutes at room temperature: Panel 1 included Integrin subunit CD4-PerCp-Cy5.5 (Invitrogen, Carlsbad, USA, OKT4), b7-PE (Biolegend, San Diego, USA, clone FIB27), CD38-APC (Biolegend, San Diego, USA, HIT2), HLA-DR-PeCy7 (Invitrogen, Carlsbad, USA, LN3), CD45RA-Bv421 (Biolegend, San Diego, USA, HI100), and CD27-APC-H7 (BD Biosciences, San Jose, USA, M-T271) while panel 2 included CD4-PerCp-Cy5.5 (Invitrogen, Carlsbad, USA, OKT4),

CD45RA-Bv421 (Biolegend, San Diego, USA, HI100), CD195 (CCR5)-APC (Miltenyi Biotech, Bergisch Gladbach, Germany, REA245), CD25-Bv605 (Biolegend, San Diego, USA, BC96), and CD8-V500 (BD Biosciences, San Jose, USA, RPA-T8). Then, 1x BD FACS™ lysing solution (BD Biosciences, San Jose, USA) was added to each of the tubes and incubated for 10 minutes at room temperature, with 2 brief vortexes during the incubation to further mix the samples. Following the lysis was a 5 minute room temperature centrifugation at 600g before 37.5 µL 100% Fetal Calf Serum (FCS, Sigma-Aldrich, Munich, Germany) was added to each tube. Finally, 250 µL of pre-chilled freezing media (12% Dimethyl Sulfoxide/FCS, both from Sigma-Aldrich, Munich, Germany) was slowly added to each tube and then the cells were transferred to Cryotubes (Thermo Scientific, Waltham, USA) for further storage. These tubes were first put into a StrataCooler Cryo Preservation Module (Agilent Technologies, Santa Clara USA) for 24 hours at -20°C. After this time, the cells were then taken out of the StrataCooler and placed directly into the liquid nitrogen gas phase for 24 additional hours. Finally, they were transferred to the liquid nitrogen liquid phase for long-term storage and transportation to a laboratory containing a 13-channel CytoFLEX (Beckman Coulter, Brea, USA).

2.2.2 Intracellular staining and image acquisition

After the cryopreserved samples were transported, they were first placed into a 37°C water bath for ~30 seconds, or until they had started to thaw slightly but not completely. Next, 2.5 mL of pre-warmed thawing media (RPMI 1640 Medium GlutaMAX supplement (Invitrogen, Carlsbad, USA) with 10% FCS (Sigma-Aldrich, Munich, Germany), 1% Penicillin-Streptomycin (Sigma-Aldrich, Munich, Germany), and 0.2 µL/mL Benzonase® Nuclease (25 U/µL, Merck Millipore, Kenilworth, USA)) was slowly added to the cells. Samples were then transferred into new 5 mL flow cytometry tubes, centrifuged for 5 minutes at 400g, and again washed with 2.5 mL pre-warmed thawing media with an additional centrifugation as above. Then, 37.5 µL FCS was added to the tube followed by 1 mL of an eBioscience™ Fixation/Permeabilization Concentrate and Diluent solution (Invitrogen, Carlsbad, USA, diluted 1:4). The cells were incubated at room temperature for 25 minutes before another centrifugation for 5 minutes at 600g. Following

the centrifugation, 2 mL of 1x eBioscience™ Permeabilization Buffer (Invitrogen) was added and the centrifugation was repeated. Following this, the intracellular antibodies were added and cells were incubated for 30 minutes at room temperature: Both panels included CD3-ECD (Beckman Coulter, Brea, USA, UCHT1) with panel 2 also including Tbet-PE (eBioscience, Frankfurt, Germany, eBio4B10), FoxP3-AF488 (Biolegend, San Diego, USA, 259D), and Eomes-PeCy7 (Invitrogen, Carlsbad, USA, WD1928). Next, again 2 mL of 1x eBioscience™ Permeabilization Buffer was added and centrifuged as above, before finally adding 200 µL of 1x BD CellFIX (BD Biosciences) to each tube. Cells were then acquired on the 13-channel CytoFLEX machine.

2.2.3 Live/dead parameter analysis

The protocol listed above was used in all participant samples in Ghana and Tanzania, along with all initial testing protocols. However, these samples did not include a live/dead parameter which was further tested in whole blood from (n=4) healthy controls. The FC panel for testing was as follows: CD4-PerCp-Cy5.5 (Invitrogen, Carlsbad, USA, OKT4), CD3-ECD (Beckman Coulter, Brea, USA, UCHT1), CD38-APC (Biolegend, San Diego, USA, HIT2), HLA-DR-PeCy7 (eBioscience, Frankfurt, Germany, LN3), and a LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, USA).

2.2.4 PBMC flow cytometry panel and protocol

To prepare the PBMCs, EDTA tubes of venous blood were taken and transported cooled (4-8°C) to the field laboratory. The PBMCs were then promptly isolated upon arrival in the laboratory. First, 7 mL EDTA blood was transferred to a 50 mL Leucosep tube containing Ficoll (Greiner Bio-One, Frickenhausen, Germany). Following a 20 minute centrifugation at 800g without a brake, the cell layers were carefully removed. They were then washed twice with sterile PBS and centrifuged for 8 minutes at 400g. Next, the cells were resuspended in RPMI 1640 medium (PAA, Linz, Austria) that was supplemented with 10% FCS (PAA) and 50 µg/mL gentamicin (PAA) followed by counting the cells with trypan blue (Sigma-Aldrich, Munich, Germany). Cells were then cryopreserved and transported in liquid nitrogen to any lab containing a CytoFLEX, as mentioned above. In this case, the cells

were taken to a laboratory in Germany where they were thawed in a 37°C water bath and washed twice with RPMI 1640 medium supplemented with 10% FCS, gentamicin, penicillin/streptomycin (all 50 µg/mL) and L-glutamine (292.3 µg/mL) (Sigma-Aldrich). Cells were then permeabilized with the FoxP3 Fixation/Permeabilization kit (Thermo Fisher Scientific). Next, all of the following intracellular antibodies were added to and incubated at 4°C for 20 minutes: CD39-BV 510 (clone TU66), CD8-BUV395 (clone HIT8a), CD4-BUV661 (clone SK3) (all obtained from BD™ Biosciences), IL-10-PE (JES3-9D7), IFN-g-FITC (clone 4S.B3), Eomes-PE-eFluor 610 (clone WD1928), TNF-α-APC (clone Mab11), Tbet-PE-Cy7 (clone 4B10), LAG-3-eFluor 450 (clone 3DS223H), PD-1-APC-eFluor 780 (clone eBoJ105), KLRG-1-PerCP-eFluor 710 (clone 13F12F2), CD127-AF700 (clone eBioRDR5), and Tim-3-Super Bright 600 (clone F38-2E2). The cells were subsequently washed with the permeabilization buffer before being resuspended in 100 µL PBS. Thermo Fisher Scientific (Life Technologies Corporation, Grand Island, USA) supplied all media, reagents, and antibodies unless otherwise specified.

2.2.5 Acquisition and analysis

All samples were acquired on the 13-channel CytoFLEX, as mentioned previously. Prior to sample acquisition, compensation was performed with the VersaComp Antibody Capture Beads (Beckman Coulter). Parameters were set so each sample acquired up to 100,000 $CD4^+$ T cell events. Image analysis was done with Flowjo_v10.6.0 (FlowJo LLC, TreeStar Inc., Ashland, USA). Statistical analysis was done with Microsoft Excel (2013, Redmond, USA), GraphPad Prism (version 6.0, GraphPad Inc., San Diego, USA), CRAN R 3.6.2, and SPSS software (IBM SPSS Statistics 22; Armonk, NY). All the variables showed non-parametric distribution by the Kolmogorow-Smirnow test and therefore, the Kruskal-Wallis test was used for multiple comparisons and followed by Dunn's post hoc test if significant ($p<0.05$).

3. Results

3.1 Gating strategy and panel design

Common protocols were used to design the FC panels. Initial method design and FC panel testing involved optimizing antibody concentration with titration testing and by using FMO controls. FC Panel 1 was designed to describe a variety of T-cell types; this included discriminating between naive, central, and effector memory cells (CD45RA, CD27), those expressing HIV receptor (α4β7), and highly activated $CD4^+$ and $CD8^+$ T cells (HLADR, CD38). FC Panel 2 was designed to identify a different set of cells; this included describing regulatory $CD4^+$ T cells (FoxP3, CD25), transcription factors used to identify exhausted cells or those under chronic inflammation (Eomes, Tbet), and an HIV entry receptor (CCR5). By using both Panel 1 and Panel 2 together, a large amount of information can simultaneously be derived from participant samples. In both of these FC panels, CD3 was added after permeabilization rather than before. This is a commonly used method that has been reported in other protocols and leads to equivalent results in our testing (112-114).

3.1.1 Comparison of cryopreservation methods

PBMC processing and cryopreservation at -80°C is the procedure commonly used in many laboratories. Many cryopreservation methods include the use of a StrataCooler (or other similar equipment) which lowers the temperature of the cells slowly, thus providing an ideal environment for cells (115). While this is best for the cells, it is often difficult to use in field laboratories or rural areas where access to -80°C freezers can often be limited; however, many of these areas are able to utilize a -20°C freezer which can be used for shorter periods of cryopreservation (116). At this time, it is also not possible to slowly freeze cells in liquid nitrogen. The StrataCooler and other such modules are not equipped for such low temperatures, due to the material they are made with, in addition to lack of space for such large containers in the portable liquid nitrogen tanks. When cells are frozen directly in the liquid nitrogen without slow freezing beforehand, there

is a greater risk of intracellular ice formation in the cells (117). Taking all of these factors into account, we were able to test the whole blood method against a variety of common cryopreservation methods. Figure 11 displays a representative sample which was prepared and cryopreserved in 4 different ways: First, the staining protocol was done with fresh whole blood (Figure 11A), the control for this method which should produce the optimal results. Next, the protocol was used and blood samples were cryopreserved in the StrataCooler at -80°C (Figure 11B), at -20°C (Figure 11C), or the novel whole blood method presented here of initially freezing with the StrataCooler at -20°C for 24 hours and then storage in liquid nitrogen (Figure 11D). In a field laboratory, this would be a feasible time frame to process cells and transport them to a main laboratory.

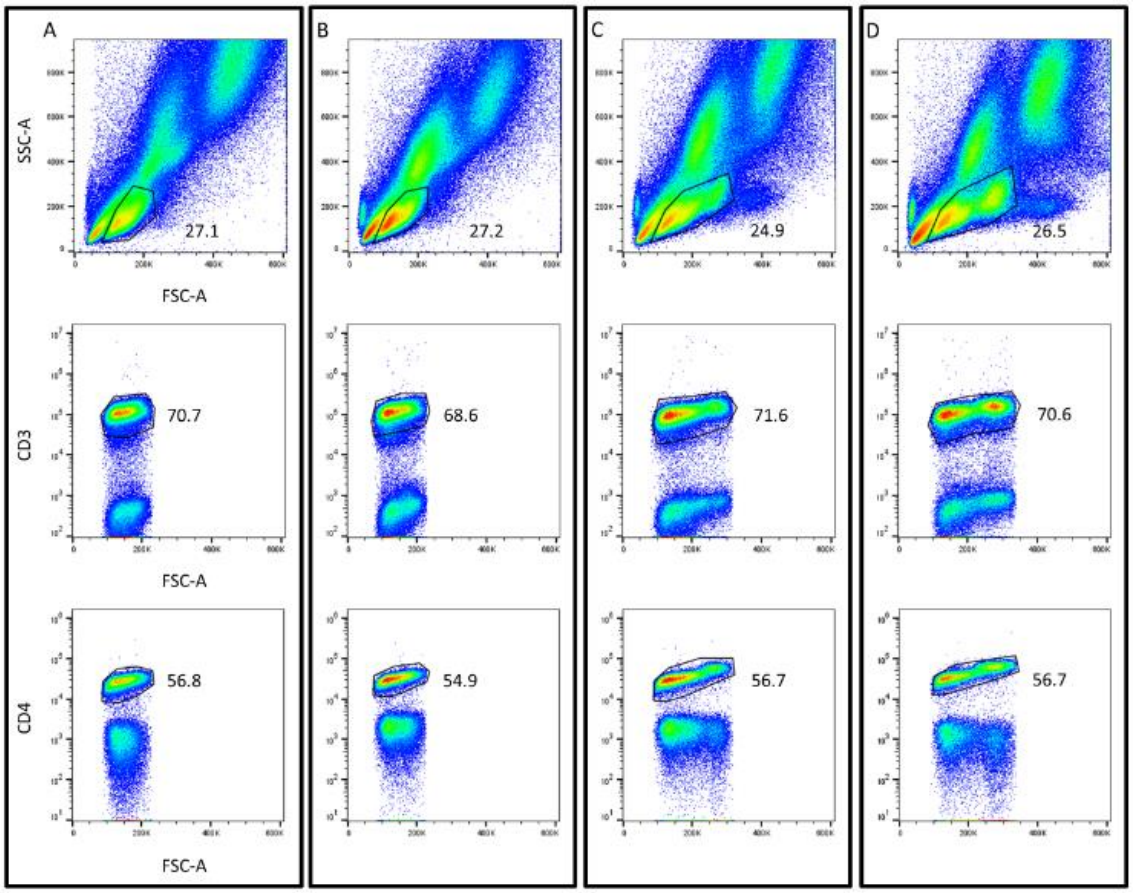


Figure 11: Representative gating of whole blood method shown using a number of cryopreservation approaches.

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As seen in Figure 11, there were similar $CD3^+$ T cell frequencies (middle row) seen in all of the conditions: A (standard method of fresh blood, mean plus standard error of the mean (SEM) 70.7±1.9), B (frozen at -80°C, 68.6±1.7), C (frozen at -20°C, 71.6±2.3), and D (frozen -20°C to LN, 70.6±2.2). The $CD4^+$ T cell frequencies were also comparable between the methods: A (56.6±0.9), B (56.7± 0.8), C (55.2±1.2), and D (55.8±0.7). Of note are the recognizable cell morphology differences that are seen in the lymphocyte populations between the fresh whole blood and any cryopreserved cells (top row). With the 2 cryopreservation methods which included first freezing cells at -20°C (Figure 11C, 11D), there is a change where it seems the lymphocyte population divides into two separate populations which is not observed in either fresh whole blood or when cryopreserved at -80°C (Figure 11A, 11B). All 4 of the healthy control samples tested and most of the participant samples using this method have displayed this so-called double lymphocyte population. This population was examined with further experiments to confirm that these cells were not monocytes (by CD3) expression or composed of dead cells.

3.1.2 Double lymphocyte population

In order to confirm if this double lymphocyte population seen after cryopreservation at -20°C was composed of monocytes (based on CD3 expression), dead cells, or clumped lymphocytes, we used healthy control samples (n=4) to do further testing. The FC panel used was previously described (2.2.3) and included CD3-ECD, CD4-PerCpC5.5, CD38-APC, HLA-DR-PeCy7, along with the addition of the LIVE/DEAD^{TM} Fixable Aqua Dead Cell Stain Kit. A representative sample of what was seen during testing is shown in Figure 12; as indicated in Figure 11D, all healthy control samples displayed this characteristic double population. Here, analysis was first done on the complete lymphocyte population (Figure 12A), the population which was left-gated (Figure 12B), and the population which was right-gated (Figure 12C). Of note, the lymphocyte frequencies within the left-gated population was noticeably higher (14.6%, mean and SEM 8.8±2.8) as compared to the right-gated population (4.47%, 7.2±1.3) (Figure 12 B, 12C, respectively). It should also be noted that in the right-gated population, there was also a higher frequency of dead cells, as seen by the LIVE/DEAD^{TM} stain, when compared to

either the left-gated population or the complete lymphocyte population (2.49 in panel C when compared to 1.33 in panel A or 0.97 in panel B). The right-gated population also displayed differences in the frequencies of the $CD3^+$ and $CD4^+$ populations when compared to either the left-gated population or the complete lymphocyte population (75.5 in panel A, 72.9 in panel A). What is especially notable is that while there were some differences seen in the $CD4^+$ T cell frequencies between figures 12A and 12B (59.9%, 62.9±1.9 in 12A and 63.3%, 72.2±4.9 in 12B), this did not affect the downstream analysis of $HLADR^+/CD38^+$ activation of $CD4^+$ T cells (2.63, 4.2±0.6 and 2.68, 4.4±0.7 for 12A and 12B, respectively). These results instill confidence in the method and that the double lymphocyte population does not affect the downstream analysis.

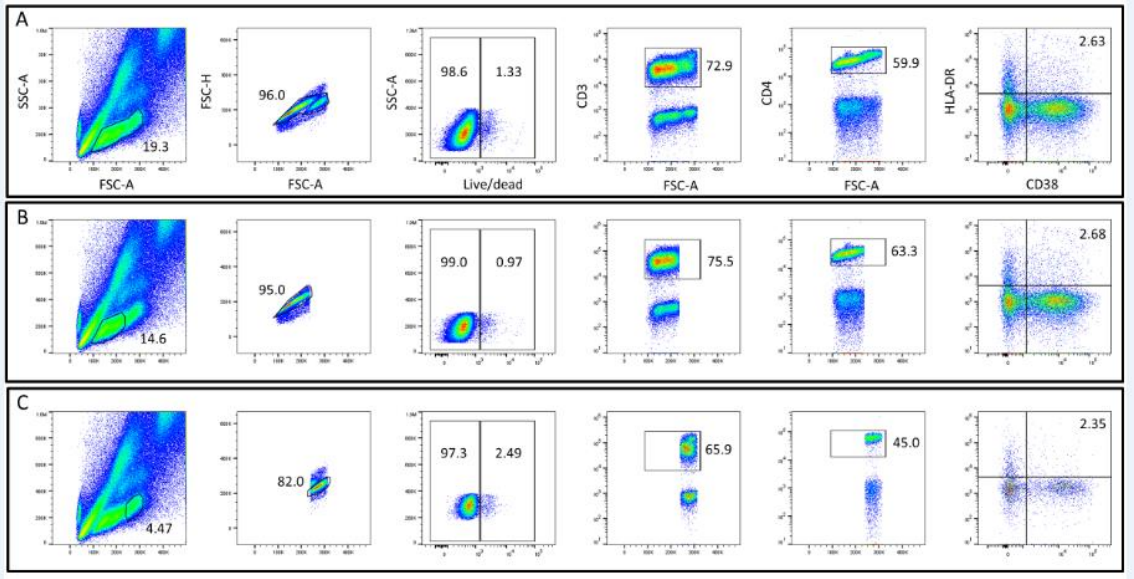

Figure 12: Investigation of the observed double lymphocyte population after cryopreservation from -20°C to liquid nitrogen.
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3.1.3 Comparison of extracellular markers

To further confirm that downstream analysis is not compromised by the cryopreservation process, a number of extracellular markers were compared between fresh whole blood and cryopreserved blood (-20°C to LN) using a representative sample (n=4). As shown and discussed in figure 12, the lymphocytes in fresh whole blood (Figure 13A) developed into the double lymphocyte population in the

cryopreserved sample (Figure 13B). As was also seen in Figure 11, the distribution of cells in the FSC/SSC plot does differ slightly between the fresh and cryopreserved -20°C to LN cells without affecting the downstream analysis of markers (e.g. HLADR, CD38). The $CD4^+$ T cell frequencies change only slightly between the fresh whole blood (85.2% (mean and SEM 63.8±0.9)) and the cryopreserved cells (84.2% (62.0±0.7)) (Figure 13C, 13D). The downstream analysis was performed to find the frequencies of $HLADR^+/CD38^+$ cells on $CD4^+$ T cells; found to be 4.36% (5.0±1.1) on fresh whole blood and 4.28% (5.3±2.1) on cryopreserved cells (Figure 13E, 13F). Additionally, the frequencies of CCR5 on $CD3^+$ T cells were observed to remain similar with frequencies of 15.3% (20.9±0.9) and 15.5% (20.9±1.0) on fresh whole blood samples and cryopreserved samples, respectively (Figure 13G, 13H). The results indicate that the method of cryopreserving whole blood at -20°C and subsequent storage in liquid nitrogen can be a valuable method to use for sophisticated flow cytometric analysis; this method produces results which are highly comparable to what is seen in fresh whole blood.

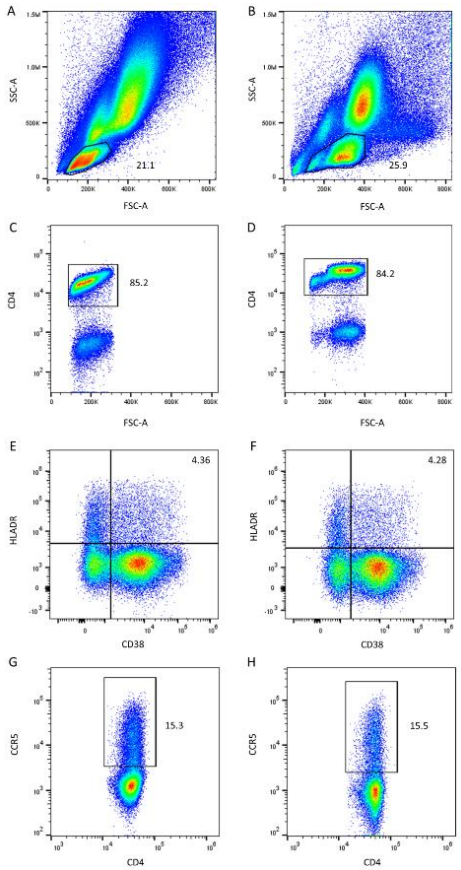

Figure 13: Comparison of extracellular makers between fresh whole blood and cryopreserved blood samples.

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3.1.4 Long-term cryopreservation effect on cells

After this method was established on healthy control samples, we could then use it on study participant samples in an ongoing Ghanaian study and test how long the samples could be stored after transportation (Figure 14). These samples were processed in the above-mentioned manner in a Ghanaian field laboratory - extracellular staining was performed followed by lysing and freezing - prior to long-term storage in liquid nitrogen. The samples were then able to be transported to a central laboratory for further processing. All of the samples were stained with the FC panel listed here and which was previously described (Appendix B). Some of these samples were processed immediately following transportation (time point 0), others were stored in liquid nitrogen for 6 months, while the rest were stored in liquid nitrogen for 12 months. After processing and image acquisition, we could compare the mean frequencies and SEM of the $CD8^+$ T cells after 0 (n=11), 6 (n=26), and 12 (n=48) months (33.06%±4.40, 28.64%±2.70, and 30.7±1.59, respectively) (Figure 14A) and $CD4^+$ T cells after 0 (n=9), 6 (n=32), and 12 (47) months storage in liquid nitrogen (62.435±2.92, 59.2%±1.93, and 59.30%±1.76, respectively) (Figure 14B). We additionally found highly comparable mean frequencies of regulatory $CD4^+$ T cells after 6 (n=20) and 12 (n=22) months storage (2.07%±0.17) and 2.54%±0.25, respectively) (Figure 5C). Further analysis of CCR5 expression on regulatory $CD4^+$ T cells after 6 (n=20) and 12 (n=22) months storage revealed no statistically significant differences between the mean frequencies (62.75%±3.18 and 63.53±3.03, respectively) (Figure 14D). None of the time points mentioned above were found to be statistically significantly different when analyzed with either Kruskal-Wallis or Mann-Whitney tests.

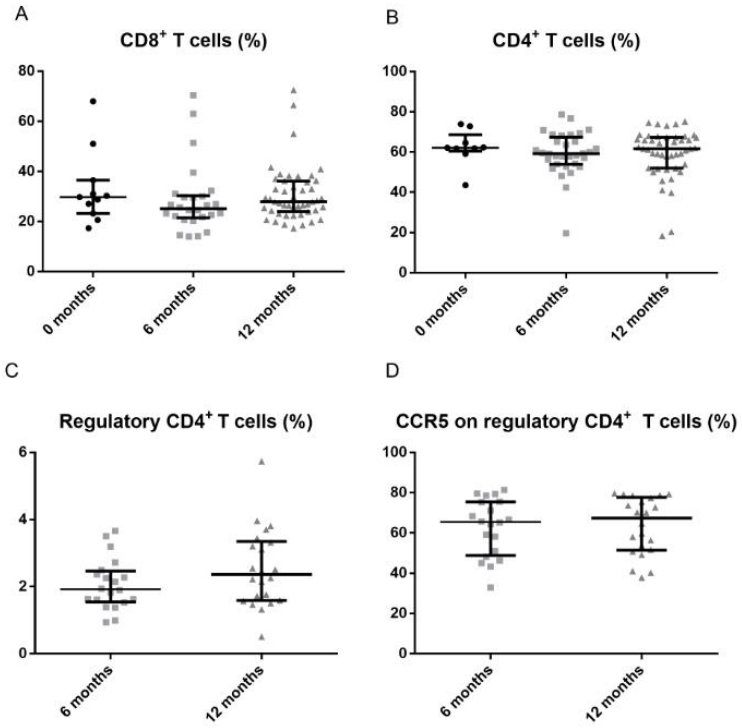

Figure 14: Whole blood method cryopreservation sample variability after storage of samples for 0, 6, or 12 months in liquid nitrogen.
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3.2 CD4⁺ T cell activation changes with increasing filarial lymphedema stage

After the initial testing of the method in Ghanaian participant samples, we were then able to employ the use of it for flow cytometric analysis in further studies in Ghana as well as start using it in Tanzania to answer specific research questions. First, we wanted to examine how the level of $CD4^+$ T cell activation differed in the various stages of filarial leg lymphedema according to the Dreyer scale (37) as part of the LEDoxy ongoing clinical trial. Participants were divided into 3 groups: Stages 1-2 (n=14), stage 3-4 (n=20), and stages 5-7 (n=25). Participant samples from Ghana showed a statistically significant decrease in the mean and SEM of $CD4^+$ T cells of the higher stage LE samples when compared to those in either of the lower stage groups (63.05%±1.74, 63.39%±1.16, and 57.83%±1.63 on groups 1-2, 3-4, and 5-7, respectively) (Figure 15A). When looking to the frequencies of the activation markers ($HLADR^+/CD38^+$) on $CD4^+$ T cells, a distinct pattern

emerges. There are statistically significant increasing frequencies seen in the upper LE stages (5-7) when compared to the lower (1-2) and moderate (3-4) groups (2.80%±0.24, 2.58%±0.19, and 4.38±0.55 on groups 1-2, 3-4, and 5-7, respectively) (Figure 15B). There were no significant differences in the frequencies of $CD4^+$ T cells in participant samples from Tanzania (stages 1-2, n=27; stages 3-4, n=39; stages 5-7, n=27) (Figure 15C); nonetheless, downstream activation markers showed increased frequencies within the higher LE stages (53.7%±2.22, 51.34%±1.59, 50.29%±1.77 on groups 1-2, 3-4, and 5-7, respectively).

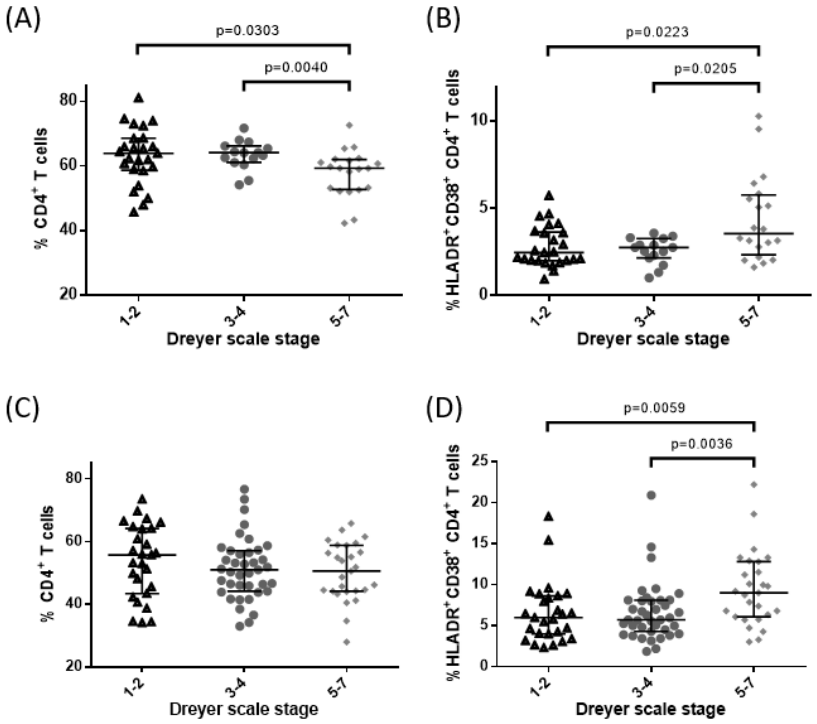

Figure 15: $CD4^+$ T cell activation in Ghanaian and Tanzanian leg lymphedema samples based on Dreyer classification stages.

3.2.1 Increased $CD4^+$ T cell activation in Ghanaian lymphedema participants

Next, we examined whether the LE participants (n=20) expressed varying levels of $CD3^+$, $CD4^+$, or activated $CD4^+$ T cells in comparison with other individuals living within the filarial endemic areas, namely endemic normals (EN. n=34) and *W. bancrofti* infected (Wb-inf., n=10) individuals. We found that while there were essentially no differences in the frequencies of the $CD3^+$ and $CD4^+$ T cells among these groups (Figure 16A, 16B), there were statistically significant differences

found when looking at the frequencies of activation markers on $CD4^+$ T cells. Here, the LE group expressed higher $CD4^+$ T cell activation levels than either the EN or Wb-inf. group, but was statistically significant only when compared to the EN group (mean and SEM, 6.08%±0.44, 5.65%±0.60, 8.35%±1.03 in the EN, Wb-inf. and LE groups, respectively) (Figure 16C).

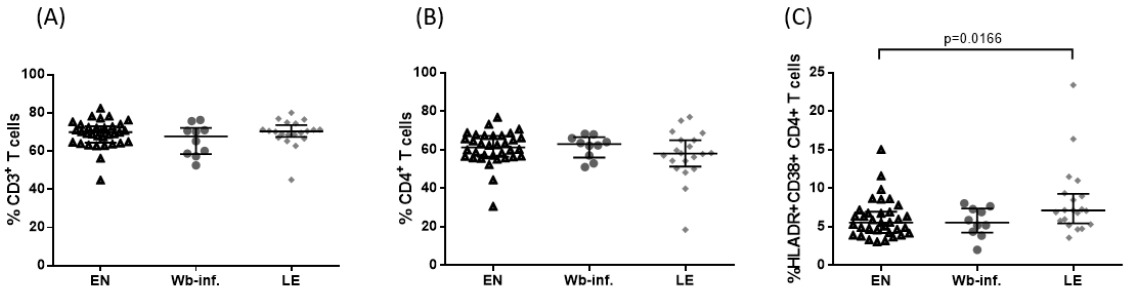

Figure 16: $CD4^+$ T cell activation in endemic normal (EN), Wuchereria bancrofti*-infected (Wb-inf.), or suffering from lymphedema (LE) pathology Ghanaian participants.*

3.3 Altered expression of $CD8^+CCR5^+CD45RA^-$ in lymphedema participants

There have not been any clinical studies which have comprehensively described exhausted $CD8^+$ T cells in various states of *W. bancrofti* infection. Using the same 3 groups mentioned above (EN, Wb-inf. and LE), we were able to use the whole blood method to examine $CD8^+$ T cells for any difference in extracellular markers (CCR5, CD45RA) which have previously been associated with memory $CD8^+$ T cells (118) (Appendix C). Upon first look, we did not see any differences *per se* in the proportion of $CD8^+$ T cells between the 3 groups (Figure 17A). However, when looking further at the $CD8^+CCR5^+$ T cells, there were significantly higher frequencies expressed in the LE patients as compared to the EN group (Figure 17B). Upon further investigation, it was seen that there was more expression on memory ($CD8^+CCR5^+CD45RA^-$) but not naive ($CD8^+CCR5^+CD45RA^+$) T cells in the LE group (Figures 17C, 17D, respectively). This suggests that the $CD8^+CCR5^+CD45RA^-$ memory T cells could potentially play a role in the development of chronic LE pathology.

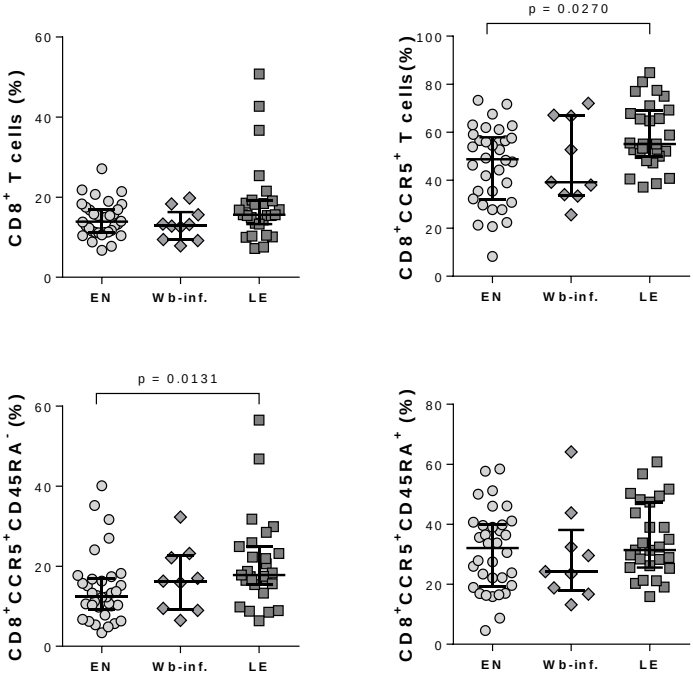

Figure 17: Participants with Wuchereria bancrofti*-associated lymphedema showed greater frequencies of CCR5⁺CD45RA⁻ expressing CD8⁺ T cells.*

3.3.1 Comparable levels of exhaustion markers in whole blood and PBMC samples

In order to confirm the whole blood method produced comparable FC results to PBMCs in a field laboratory setting, a subset of Ghanaian participant samples (EN, n=11; Wb-inf., n=8; LE, n=8) were tested with both methods (Figure 18). The whole blood was stained with the FC panel above (Appendix B) while the PBMCs were stained with a 13-color FC panel designed to include more parameters related to $CD8^+$ T cell exhaustion. The markers Tbet and Eomes were chosen as a main component of the FC panel due to the fact that Tbet is important for the expression of effector functions and Eomes is the gatekeeper for the memory $CD8^+$ T cell repertoire (119). The expression levels of these transcription factors, Tbet and Eomes, in $CD8^+$ T cells can provide further information about progenitor and terminally exhausted subsets (89, 93, 120, 121). We then compared the frequencies of $CD8^+$ T cells (Figure 18A, 18B), $CD8^+Ex_{mem}$ cells (defined as $Tbet^{dim}Eomes^{hi}$, Figure 18C, 18D), and $CD8^+Ex_{eff}$ cells (defined as $Tbet^{hi}Eomes^{dim}$, Figure 18E, 18F) (89). While there were no significant differences found between either the $CD8^+$ or the $CD8^+Ex_{mem}$ cells, there was a pattern

observed with the $CD8^+Ex_{eff}$ cells. While the LE group produced significantly higher frequencies compared to the Wb-infected within the whole blood method (Figure 18E, p=0.0064), the same trend towards an increase in the LE group was observed in the PBMC samples, albeit insignificant (Figure 18F, p=0.0613). Given the low number of overlapping samples, the methods are shown to be comparable and can be further replicated with a greater number of samples.

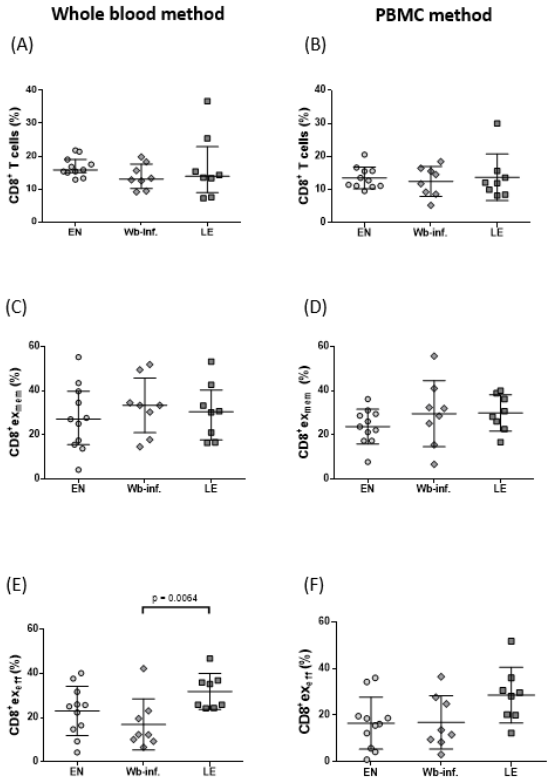

Figure 18: Comparison of $CD8^+ex_{mem}$ and $CD8^+ex_{eff}$ populations between whole blood and PBMC methods.

3.3.2 Decreased frequencies of IFN-γ in $CD8^+ex_{mem}$ T cell subsets of lymphedema participants

$CD8^+$ T cell exhaustion occurs when antigen is present over a long period of time or there is inflammation which leads to the cells not being able to function effectively. This is shown by the increased frequencies of certain surface markers (e.g. PD-1, Tim-3) and elevated plasma levels of IL-10 (122). Because of this, we compared the expression levels of $CD8^+ex_{mem}$ T cells that were displaying exhaustion markers LAG-3, Tim-3, PD-1, KLRG-1, and CD39 in addition to IFN-γ, IL-10, and TNF-α expression using the gating strategy in Appendix D. The initial comparison

of $CD8^+ex_{mem}Tim\text{-}3^+$ was similar between the 3 groups (Figure 19A), reduced frequencies of $CD8^+ex_{mem}Tim\text{-}3^+IFN\text{-}\gamma^+$ cells was seen in the LE group in comparison to both EN and Wb-infected (Figure 19B). There was a similar reduction in the median fluorescence intensity (MFI) in the LE group as compared to the Wb-infected, indicating decreased IFN-γ production by the LE group (Figure 19C). Additionally, there was a trend towards a further decrease in $CD8^+ex_{mem}Tim\text{-}3^+IFN\text{-}\gamma^+$ with increase LE stage (between stages 2, 3, and 6), although insignificant due to low sample numbers. It was quite notable to find that while there were comparable frequencies of $CD8^+ex_{mem}LAG\text{-}3^+$ and $CD8^+ex_{mem}LAG\text{-}3^+IFN\text{-}\gamma^+$ (Figure 19D, 19E, respectively) between the groups, the MFI was reduced in the LE groups when compared to the other 2 subsets (Figure 19F). This is indicative of decreased activity of the cells in this group. The other markers measured (CD39, PD-1, and KLRG-1) did not display any significant changes between the groups.

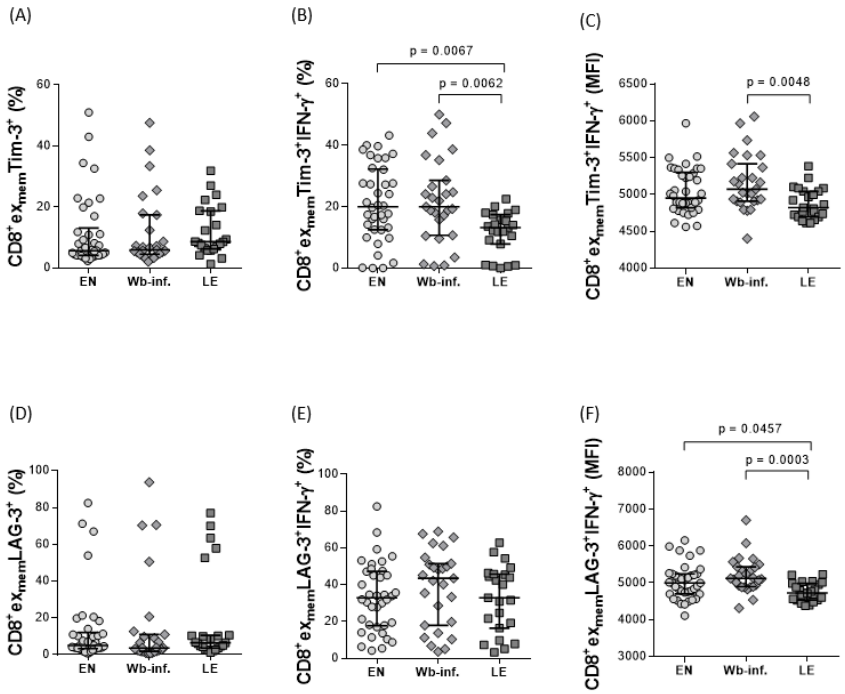

Figure 19: $CD8^+ex_{mem}$ T cell subsets of lymphedema individuals display lower frequencies of IFN-γ in cells expressing Tim-3 or LAG-3.

3.3.3 Increased expression of IL-10 in CD8$^+$ex$_{eff}$ T cell subsets of lymphedema participants

We then wanted to further analyze the CD8$^+$ex$_{eff}$ subset as well to find any differences in the exhaustion markers or cytokines. Interestingly, Tim-3 expressing cells again displayed distinct patterns, but in these cells the LE group expressed significantly higher frequencies of Tim-3 on CD8$^+$ex$_{eff}$ cells (Figure 20A). Frequencies of CD8$^+$ex$_{eff}$Tim-3$^+$IL-10$^+$ expressing cells were also higher in the LE group as compared to the EN group (Figure 20B). However, the CD8$^+$ex$_{eff}$Tim-3$^+$IL-10$^+$ MFI was consistent across all 3 groups (Figure 20C). In terms of the LAG-3 expression on the CD8$^+$ex$_{eff}$ subset, there were no differences seen (Figure 20D). When examining this population on IL-10 producing cells, levels were significantly elevated within the LE group as compared to the EN group, however this was not seen in the MFI of the same group (Figure 20E, 20F, respectively). Within the CD8$^+$ex$_{eff}$ subset, we continued to see increased frequencies of CD8$^+$ex$_{eff}$IL-10$^+$ cells in the LE cohort when looking at the expression of CD39, KLRG-1, and PD-1 (Figure 21A, 21B, and 21C, respectively).

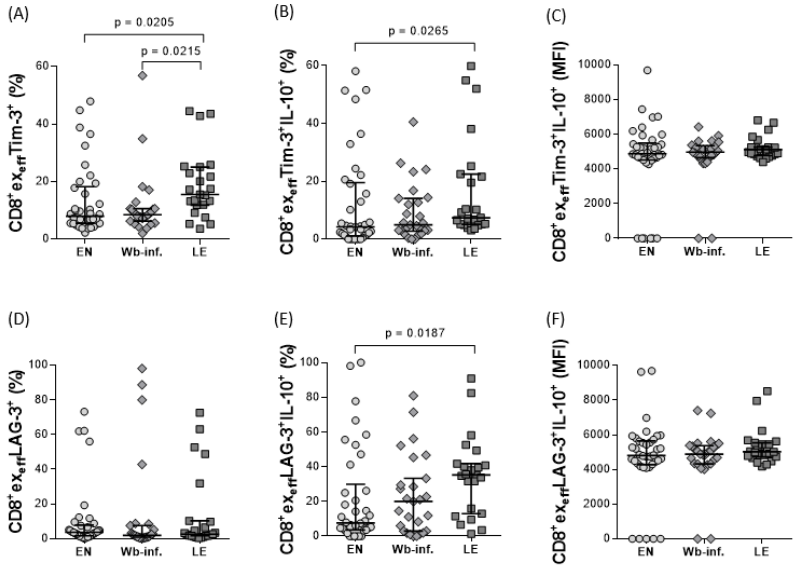

Figure 20: CD8$^+$ex$_{eff}$ T cell subsets of lymphedema individuals present higher frequencies of IL-10 in cells expressing Tim-3 or LAG-3.

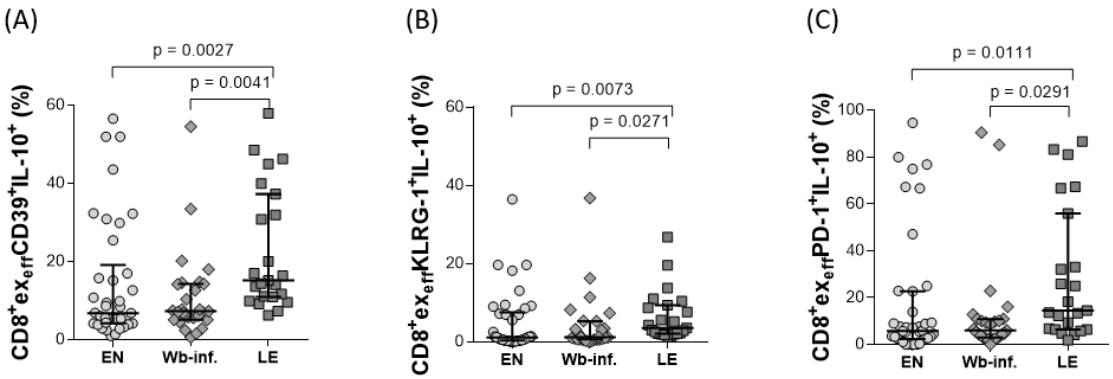

Figure 21: Lymphedema participants present increased frequencies of IL-10 in CD39⁺, KLRG-1⁺, and PD-1⁺ in the CD8⁺ex$_{eff}$ subsets.

4. Discussion

There are ~40 million people currently suffering from LE worldwide with no sufficient treatment to cure chronic LE. While MDA programs have significantly reduced the number of newly infected individuals, the numbers of people with irreversible LE are unlikely to change (123). It is therefore important to understand how the immune system is being modified by the *W. bancrofti* itself in those who suffer from LE along with those who are infected and asymptomatic. In addition, these individuals can be subject to other bacterial infections and resulting ADLA from when bacteria enters through open lesions (39, 123). Since the higher stages of LE are associated with a greater number of open lesions, ADLA is commonly associated with more progressed LE stages.

4.1 Field laboratory obstacles

In order to address LF and other infections studied in hard to reach areas, a FC method was needed to overcome issues like the lack of standardized equipment (e.g. -80°C freezer, incubators, Laminar flow hood) or alternatively, equipment which undergoes harsher wear and tear than in a standard laboratory environment (98). These laboratories can be located in areas with higher humidity or lack of climate controlled rooms to store the equipment leading to quicker breakdown. There often aren't technicians trained to repair the equipment or perform quality control checks on a regular basis; when machines do break down or need repair, the parts can be difficult to obtain or take long periods of time to reach the laboratories. Despite these problems, there is a vast potential for research in these areas as well as for capacity building activities. NTDs are often located in these areas which only have access to field laboratories; hence, there is a need for standardized procedures which allow researchers to easily obtain high quality data.

A typical field procedure is to use PBMCs to preserve samples for later flow cytometric analysis (98, 100, 103). Due to some of the above mentioned problems with PBMC processing, we designed a novel whole blood method which can be used to accurately characterize a number of extra- and intracellular markers

measured with flow cytometry; the method utilizes a smaller amount of blood (~200 µL per participant) and avoids time-consuming centrifugation steps (to avoid effects of power supply problems). In addition to the centrifugation step which can be interrupted by irregular power supplies, deep freezing with a -80°C freezer and long-term sample storage can also be impacted. To overcome this, the whole blood method was designed to instead include an intermittent freezing step at -20°C (freezers at this temperature are more readily available in limited resource settings) for 24 hours (or until completely frozen) before transfer to a portable liquid nitrogen tank. While the extracellular marker panel does need to be fixed before using in the field, the intracellular marker panel can be added to and modified in order to address ongoing research questions.

Pinto and colleagues also noted the lack of appropriate FC field methods and tested 5 variations of fixing and freezing methods, one of which was similar to our method of staining, fixing, and freezing (in that particular order) (110). Unfortunately, this group only tested the method on 5 markers (CD3, CD4, CD9, CD19, and CD16/CD56), and of these, CD3, CD4, and CD8 can also be stained after fixation, freezing, and permeabilization and are not shown to be sensitive to fixation (110, 124-126). Some extracellular epitopes, notably HLADR which is used to define activated $CD4^+$ and $CD8^+$ T cells, are greatly affected by the paraformaldehyde fixation step, making it difficult to reliably use if stained for after fixation (107-109).

4.2 $CD4^+$ T cell activation

Helminth immune modulation of a host's immune system has been thoroughly documented; the overall goal of the helminth is to reproduce and prevent expulsion from the host (56, 127-130). Due to the nature of *W. bancrofti* infections, infected individuals can either be asymptomatic or suffer from a chronic LE (or hydrocele) infection. Studies have well characterized the varying $CD4^+$ T cell characteristics between filarial endemic individuals and those suffering from LE. Among the reports are elevated TNF receptor frequencies (131), variety of inflammatory markers present in the peripheral circulation (TARC, IP-10, MCP-1, IL-6, TNF-α, and C-reactive protein) (61), and higher levels of Th1, Th17, Th9,

with simultaneous lower levels of the Th2 response seen in filarial lymphedema cohorts (65).

Previous studies have shown a significant link between systemic immune activation and helminth infections; Chachage *et al* found increased frequencies of activated T cells in *Trichuris trichiura* and *Ascaris lumbricoides* infections whereas Kroidl *et al* found the same association with *W. bancrofti* infections (78, 132). Another group studied Ethiopian immigrants in Israel and found that the more recent immigrants, when compared to the immigrants who had lived there for more than 5 years, displayed increased levels of $HLADR^+$ activation on both $CD4^+$ and $CD8^+$ T cells, hypothesized to be due to chronic helminth infections being more prevalent in the recent immigrant group (70). Very few of these groups looked at the activation specifically in regards to *W. bancrofti* infection, and especially not in regards to varying stages of LE pathology. In our studies, we found the largest differences in activation between the chronic pathology LE groups when compared to the EN or the Wb-infected (asymptomatic infection) groups with a trend towards increasing activation levels and higher LE stages. It has been hypothesized since the 1990s that the systemic immune activation induced by helminth infections can contribute to host susceptibility to HIV infections (67) and was then further expanded on to show this was true specifically in *W. bancrofti* infections (79). It remains to be shown if this is indeed true for past LF infections which have already eliminated the worm, succumb to pathology, and yet remain activated (LE group).

Early response of individuals infected with *W. bancrofti* is difficult to characterize since it is almost impossible to determine the exact time of infection. Babu and Nutman tried to overcome this by exposing PBMCs to L3 larvae, showing early T cell activation levels as indicated by upregulation of CD69 and CD71 (133). Babu has also demonstrated that LE patients do not have an immune response that differs significantly from asymptomatic infections in terms of the Th2 and IL-10 responses (65). However, what we see is that the activation levels are indeed upregulated in LE chronic pathology patients when compared to either asymptomatic (Wb-inf.) or EN groups as well as displaying an increase in activation levels associated with the higher stages of LE. This is an interesting finding because by the time individuals develop pathology, antigen is typically no longer present in

detectable levels. Indeed, all the LE PBMC samples tested CFA negative with both the FTS and TropBio tests.

Over time, LE often continues to progress; in the higher LE stages, hyperpigmentation and hyperkeratosis cause the skin to become thick and hard, and the individuals are more prone to bacterial infections and ADLA (134-136). While these samples were not tested for the presence of other bacterial or viral infections, we hypothesize that this is perhaps one of the contributing factors for the higher activation levels associated with LE in general, along with the trend towards higher activation as LE stages increase. Babu and Nutman have also suggested that the immune activation could be caused since microbes have an easier time moving into the body through the broken skin caused by LE (62).

4.3 $CD8^+$ T cell exhaustion

Chronic activation of cells when they are exposed to persistent antigen can lead to a state of T cell exhaustion. We noted that the LE individuals, in addition to increased frequencies of activation markers, showed altered expression of exhausted $CD8^+$ T cells in both whole blood and PBMC samples. By first looking at the results from the whole blood method, we were able to see that the LE group expressed elevated frequencies of $CD8^+$CCR5 expressing T cells when compared to either the EN or Wb-infected groups. Upon further examination, this was found to be statistically significant also within the memory ($CD45RA^-$) subset but not within the naive ($CD45RA^+$) subset for LE participants. Until now, the role of CCR5 on $CD8^+$ T cells has not been well defined for filariasis patients. What is known, however, is that during viral infections, CCR5 plays an important role in recruiting memory $CD8^+$ T cells to areas of inflammation (118). CCR5 has also been well characterized as a co-receptor for HIV on $CD4^+$ T cells (137, 138). The inflammatory response is activated for many diseases based on the interaction of CCR5 with chemokines. While LF specific studies have not reported any altered frequencies of CCR5 on $CD8^+$ T cells, there has been a review published which reports on the interplay of CCR5 within infections by another helminth, *Schistosoma*. Here, they showed that the common thought of “lack of CCR5 is

associated with less inflammation" does not hold true in the case of schistosomiasis (139). It appears that our results also correlated to what is seen in schistosomiasis, given the higher frequencies of $CD8^+CCR5^+$ T cells in the LE group (inflammation).

Within a subset of these samples, we could confirm the validity of our newly developed whole blood method with a direct comparison to PBMC samples. With the small subset of individuals, the same trends were observed within the exhausted $CD8^+$ effector ($CD8^+Ex_{eff}$, $CD8^+Tbet^{hi}Eomes^{low}$) and memory ($CD8^+Ex_{mem}$, $CD8^+Tbet^{dim}Eomes^{hi}$) T cell groups (89), even though the LE group had significantly higher frequencies of $CD8^+ex_{eff}$ cells compared to the EN while that was not seen in the PBMC samples. Visual examination of the samples also showed a more precise marker cutoff for the whole blood samples, in this case for transcription factors Tbet and Eomes.

Given the low number of samples analyzed with both methods, it was necessary to use PBMC samples to analyze a large number of markers associated with exhaustion. Buggert *et al*. had previously showed evidence of continual expression of Eomes and PD-1 in the viral specific $CD8^+$ T cells in HIV positive individuals; they reported that the expression patterns of Tbet and Eomes together are indicative of insufficient viral clearance (89). Since they were primarily interested in the ratio of Tbet and Eomes, we expanded on this simple ratio by continuing the analysis with IL-10, IFNγ, Tim-3, LAG-3, CD39, KLRG-1, and PD-1 on $CD8^+Ex_{mem}$ and $CD8^+Ex_{eff}$ subsets within filarial infections.

What has been seen in previous studies is that simultaneous analysis of multiple exhaustion markers can display a more accurate picture of the level of exhaustion. This is what has been often seen with the markers LAG-3 and PD-1 when comparing single positive and double positive cells for these markers (140-142). Other studies have been interested in characterizing the $CD4^+$ and $CD8^+$ exhausted T cells by examining more of the exhaustion markers as well (e.g. Tim-3 and IL-10 in addition to those above) (122). Interestingly, just because cells are co-expressing multiple exhaustion markers, it does not necessarily indicate that cells are overall more exhausted; co-expression of PD-1 and Tim-3 has shown the opposite (143-145). Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4)

is another marker associated with exhaustion (not measured here); it was however previously studied in regards to filarial infections (60, 146) and the co-expression of PD-1 and CTLA-4 were reported to even suppress a Th2 response in *in vitro Strongyloides stercoralis* infections (147). As mentioned earlier, filarial infections characteristically display an upregulated Th2 response to resist developing chronic pathology (147, 148). Here, this analysis was also performed, but no conclusions could be drawn due to the low number of cells co-expressing the exhaustion markers. However, we were able to conclude that there is sufficient evidence of an altered $CD8^+$ exhausted T cell exhaustion pattern present within LE chronic pathology individuals. It was especially interesting to note that while so many studies have shown distinct immune responses to *W. bancrofti*, we did not find any differences in the exhaustion patterns of the *W.* bancrofti-infected individuals when compared to the EN individuals.

Given that the majority of LE patients have already cleared the infection, also seen in our PBMC samples where there were no antigen positive individuals, it is unlikely that the altered activation and exhaustion patterns are caused by circulating filarial antigens. A more likely cause would be the secondary viral or bacterial infections often found within LE patients and which are more concentrated within higher LE stages. Here, we were able to observe trends of increasing activation levels corresponding to increasing LE stage. This needs to be further examined with larger number of samples in each LE stage, particularly the middle stages 4 and 5.

4.4 Conclusion

There continues to be a need for research on LF due to the chronicity of the infection and the ongoing pathology (LE). We first realized there was a lack of information regarding specific flow cytometric markers (e.g. activation) based on LE staging. Our main finding here is that the immune activation increases significantly with the higher LE stages, indicating that the immune system is more active from potential viral or bacterial infections from open lesions or from the LF infection itself.

We also noticed that there had been no research done on the exhausted $CD8^+$ T cell subsets within *W. bancrofti* infections. When comparing the LE infected individuals with either the Wb-infected or control EN, we found that there were very few differences between the Wb-infected and EN, but the LE-infected displayed distinct exhaustion patterns.

While asking these questions, we noticed there was a lack of appropriate flow cytometry field methods, which is a problem encountered not just when studying filarial infections but in all resource limited settings. To solve this, we developed a novel field method in Munich, Germany which was then employed in a number of clinical trials and other studies to ask the aforementioned questions. These novel findings further support the knowledge about the immune response of individuals with both asymptomatic *W. bancrofti* infections and those suffering from chronic LE pathology.

References

1. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Invest. 2008;118(4):1311-21.

2. Al Amin ASM, Wadhwa R. Helminthiasis. StatPearls. Treasure Island (FL): StatPearls Publishing

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3. WHO. Soil-transmitted helminth infections. 2021.

4. NIAID. Neglected Tropical Diseases. 2016.

5. CDC. Neglected Tropical Diseases. 2020.

6. WHO. Lymphatic Filariasis. 2021.

7. GBD. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 2018;392(10159):1789-858.

8. Ramaiah KD, Ottesen EA. Progress and impact of 13 years of the global programme to eliminate lymphatic filariasis on reducing the burden of filarial disease. PLoS Negl Trop Dis. 2014;8(11):e3319.

9. GAELF. Progress to Elimination.

10. Clayton KL, Douglas-Vail MB, Nur-ur Rahman AK, Medcalf KE, Xie IY, Chew GM, et al. Soluble T cell immunoglobulin mucin domain 3 is shed from CD8+ T cells by the sheddase ADAM10, is increased in plasma during untreated HIV infection, and correlates with HIV disease progression. J Virol. 2015;89(7):3723-36.

11. CDC. Epidemiology and Risk Factors. 2019.

12. Stolk WA, de Vlas SJ, Habbema JD. Advances and challenges in predicting the impact of lymphatic filariasis elimination programmes by mathematical modelling. Filaria J. 2006;5:5.

13. Simonsen PE, Niemann L, Meyrowitsch DW. Wuchereria bancrofti in Tanzania: microfilarial periodicity and effect of blood sampling time on microfilarial intensities. Trop Med Int Health. 1997;2(2):153-8.

14. Card CM, Rutherford WJ, Ramdahin S, Yao X, Kimani M, Wachihi C, et al. Reduced cellular susceptibility to in vitro HIV infection is associated with CD4+ T cell quiescence. PLoS One. 2012;7(9):e45911.

15. Stolk WA, Swaminathan S, van Oortmarssen GJ, Das PK, Habbema JD. Prospects for elimination of bancroftian filariasis by mass drug treatment in Pondicherry, India: a simulation study. J Infect Dis. 2003;188(9):1371-81.

16. WHO. Global programme to eliminate lymphatic filariasis: proress report, 2019. 2019.

17. WHO. Monitoring and epidemiological assessment of mass drug administration in the global programme to eliminate lymphatic filariasis: a manual for national elimination prorammes. 2020.

18. The global distribution of lymphatic filariasis, 2000-18: a geospatial analysis. Lancet Glob Health. 2020;8(9):e1186-e94.

19. Kwarteng A, Ahuno ST, Akoto FO. Killing filarial nematode parasites: role of treatment options and host immune response. Infect Dis Poverty. 2016;5(1):86.

20. Addiss D, Critchley J, Ejere H, Garner P, Gelband H, Gamble C. Albendazole for lymphatic filariasis. Cochrane Database Syst Rev. 2004(1):Cd003753.

21. Meyrowitsch DW, Simonsen PE. Efficacy of DEC against Ascaris and hookworm infections in schoolchildren. Trop Med Int Health. 2001;6(9):739-42.

22. Taylor MJ, Hoerauf A, Bockarie M. Lymphatic filariasis and onchocerciasis. Lancet. 2010;376(9747):1175-85.

23. Fernando SD, Rodrigo C, Rajapakse S. Current evidence on the use of antifilarial agents in the management of bancroftian filariasis. J Trop Med. 2011;2011:175941.

24. Hoerauf A, Mand S, Fischer K, Kruppa T, Marfo-Debrekyei Y, Debrah AY, et al. Doxycycline as a novel strategy against bancroftian filariasis-depletion of Wolbachia endosymbionts from Wuchereria bancrofti and stop of microfilaria production. Med Microbiol Immunol. 2003;192(4):211-6.

25. Taylor MJ, Hoerauf A. Wolbachia bacteria of filarial nematodes. Parasitol Today. 1999;15(11):437-42.

26. Mand S, Debrah AY, Klarmann U, Batsa L, Marfo-Debrekyei Y, Kwarteng A, et al. Doxycycline improves filarial lymphedema independent of active filarial infection: a randomized controlled trial. Clin Infect Dis. 2012;55(5):621-30.

27. Chesnais CB, Vlaminck J, Kunyu-Shako B, Pion SD, Awaca-Uvon NP, Weil GJ, et al. Measurement of Circulating Filarial Antigen Levels in Human Blood with a Point-of-Care Test Strip and a Portable Spectrodensitometer. Am J Trop Med Hyg. 2016;94(6):1324-9.

28. Weil GJ, Ramzy RM. Diagnostic tools for filariasis elimination programs. Trends Parasitol. 2007;23(2):78-82.

29. CDC. Lymphatic filariasis diagnosis. 2018.

30. Notes M. Giemsa Stain - Principle, Procedure, Results, Interpretation. 2020.

31. WHO. Bench Aids for the diagnosis of filarial infections. 1997.

32. Weil GJ, Jain DC, Santhanam S, Malhotra A, Kumar H, Sethumadhavan KV, et al. A monoclonal antibody-based enzyme immunoassay for detecting parasite antigenemia in bancroftian filariasis. J Infect Dis. 1987;156(2):350-5.

33. Weil GJ, Lammie PJ, Weiss N. The ICT Filariasis Test: A rapid-format antigen test for diagnosis of bancroftian filariasis. Parasitol Today. 1997;13(10):401-4.

34. Gass K, Beau de Rochars MV, Boakye D, Bradley M, Fischer PU, Gyapong J, et al. A multicenter evaluation of diagnostic tools to define endpoints for programs to eliminate bancroftian filariasis. PLoS Negl Trop Dis. 2012;6(1):e1479.

35. Weil GJ, Curtis KC, Fakoli L, Fischer K, Gankpala L, Lammie PJ, et al. Laboratory and field evaluation of a new rapid test for detecting Wuchereria bancrofti antigen in human blood. Am J Trop Med Hyg. 2013;89(1):11-5.

36. More SJ, Copeman DB. A highly specific and sensitive monoclonal antibody-based ELISA for the detection of circulating antigen in bancroftian filariasis. Trop Med Parasitol. 1990;41(4):403-6.

37. Dreyer G, Addiss, D., Dreyer, P., Noroes, J. Basic Lymphoedema Management. Treatment and Prevention of Problems Associated with Lymphatic Filariasis Gerusa Dreyer, David Addiss, Patricia Dreyer & Joaquim Noroes. Hollis, NH: Hollis Publishing, 2002. x +112 pp. Price: inside USA US$5 for first book and US$1.50 for each additional copy; outside USA US$14 for first book and US$5 for each additional copy. ISBN 1-884186-17-3. Transactions of The Royal Society of Tropical Medicine and Hygiene. 2003;97(5):566-.

38. Dreyer G, Addiss D, Gadelha P, Lapa E, Williamson J, Dreyer A. Interdigital skin lesions of the lower limbs among patients with lymphoedema in an area endemic for bancroftian filariasis. Trop Med Int Health. 2006;11(9):1475-81.

39. Dreyer G, Medeiros Z, Netto MJ, Leal NC, de Castro LG, Piessens WF. Acute attacks in the extremities of persons living in an area endemic for bancroftian filariasis: differentiation of two syndromes. Trans R Soc Trop Med Hyg. 1999;93(4):413-7.

40. Olszewski WL, Jamal S, Manokaran G, Pani S, Kumaraswami V, Kubicka U, et al. Bacteriological studies of blood, tissue fluid, lymph and lymph nodes in patients with acute

dermatolymphangioadenitis (118) in course of 'filarial' lymphedema. Acta Trop. 1999;73(3):217-24.

41. Furlong-Silva J, Cross SD, Marriott AE, Pionnier N, Archer J, Steven A, et al. Tetracyclines improve experimental lymphatic filariasis pathology by disrupting interleukin-4 receptor-mediated lymphangiogenesis. J Clin Invest. 2021;131(5).

42. Ottesen EA. The global programme to eliminate lymphatic filariasis. Trop Med Int Health. 2000;5(9):591-4.

43. Wynd S, Melrose WD, Durrheim DN, Carron J, Gyapong M. Understanding the community impact of lymphatic filariasis: a review of the sociocultural literature. Bull World Health Organ. 2007;85(6):493-8.

44. Evans DB, Gelband H, Vlassoff C. Social and economic factors and the control of lymphatic filariasis: a review. Acta Trop. 1993;53(1):1-26.

45. Bandyopadhyay L. Lymphatic filariasis and the women of India. Soc Sci Med. 1996;42(10):1401-10.

46. Gyapong M, Gyapong JO, Adjei S, Vlassoff C, Weiss M. Filariasis in northern Ghana: some cultural beliefs and practices and their implications for disease control. Soc Sci Med. 1996;43(2):235-42.

47. Tracey KJ. The inflammatory reflex. Nature. 2002;420(6917):853-9.

48. Jenni Punt SS, Patricia Jones, Judith A Owen. Kuby Immunology (8th Edition): WH Freeman; 2018.

49. Thomas J Kindt BAO, Richard A Goldsby. Immunology. 6th Edition ed. New York: WH Freeman; 2007.

50. McSorley HJ, Maizels RM. Helminth infections and host immune regulation. Clin Microbiol Rev. 2012;25(4):585-608.

51. Feary J, Britton J, Leonardi-Bee J. Atopy and current intestinal parasite infection: a systematic review and meta-analysis. Allergy. 2011;66(4):569-78.

52. Greenwood BM. Autoimmune disease and parasitic infections in Nigerians. Lancet. 1968;2(7564):380-2.

53. Mutapi F, Imai N, Nausch N, Bourke CD, Rujeni N, Mitchell KM, et al. Schistosome infection intensity is inversely related to auto-reactive antibody levels. PLoS One. 2011;6(5):e19149.

54. van den Biggelaar AH, Rodrigues LC, van Ree R, van der Zee JS, Hoeksma-Kruize YC, Souverijn JH, et al. Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren. J Infect Dis. 2004;189(5):892-900.

55. Hoerauf A, Satoguina J, Saeftel M, Specht S. Immunomodulation by filarial nematodes. Parasite Immunol. 2005;27(10-11):417-29.

56. Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol. 2003;3(9):733-44.

57. Allen JE, Maizels RM. Diversity and dialogue in immunity to helminths. Nat Rev Immunol. 2011;11(6):375-88.

58. Mahanty S, Mollis SN, Ravichandran M, Abrams JS, Kumaraswami V, Jayaraman K, et al. High levels of spontaneous and parasite antigen-driven interleukin-10 production are associated with antigen-specific hyporesponsiveness in human lymphatic filariasis. J Infect Dis. 1996;173(3):769-73.

59. Sartono E, Kruize YC, Kurniawan A, Maizels RM, Yazdanbakhsh M. Depression of antigen-specific interleukin-5 and interferon-gamma responses in human lymphatic filariasis as a function of clinical status and age. J Infect Dis. 1997;175(5):1276-80.

60. Steel C, Nutman TB. CTLA-4 in filarial infections: implications for a role in diminished T cell reactivity. J Immunol. 2003;170(4):1930-8.

61. Babu S, Nutman TB. Immunopathogenesis of lymphatic filarial disease. Semin Immunopathol. 2012;34(6):847-61.

62. Babu S, Nutman TB. Immunology of lymphatic filariasis. Parasite Immunol. 2014;36(8):338-46.

63. Suswillo RR, Owen DG, Denham DA. Infections of Brugia pahangi in conventional and nude (athymic) mice. Acta Trop. 1980;37(4):327-35.

64. Vincent AL, Sodeman WA, Winters A. Development of Brugia pahangi in normal and nude mice. J Parasitol. 1980;66(3):448.

65. Babu S, Bhat SQ, Pavan Kumar N, Lipira AB, Kumar S, Karthik C, et al. Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells. PLoS Negl Trop Dis. 2009;3(4):e420.

66. UNAIDS. Global HIV & AIDS statistics - Fact sheet. 2021.

67. Bentwich Z, Kalinkovich A, Weisman Z. Immune activation is a dominant factor in the pathogenesis of African AIDS. Immunol Today. 1995;16(4):187-91.

68. Bentwich Z, Kalinkovich A, Weisman Z, Borkow G, Beyers N, Beyers AD. Can eradication of helminthic infections change the face of AIDS and tuberculosis? Immunol Today. 1999;20(11):485-7.

69. Bentwich Z, Kalinkovich A, Weisman Z, Grossman Z. Immune activation in the context of HIV infection. Clin Exp Immunol. 1998;111(1):1-2.

70. Kalinkovich A, Maayan S, Weisman Z, Harpaz N, Bentwich Z. Immune activation, a co-factor for HIV transmission in Thailand? Lancet. 1994;343(8911):1506-7.

71. Hazenberg MD, Otto SA, van Benthem BH, Roos MT, Coutinho RA, Lange JM, et al. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. Aids. 2003;17(13):1881-8.

72. Hofmann B, Nishanian P, Baldwin RL, Insixiengmay P, Nel A, Fahey JL. HIV inhibits the early steps of lymphocyte activation, including initiation of inositol phospholipid metabolism. J Immunol. 1990;145(11):3699-705.

73. Milestones. Immune activation linked to pathogenesis. 2018.

74. Bégaud E, Chartier L, Marechal V, Ipero J, Léal J, Versmisse P, et al. Reduced CD4 T cell activation and in vitro susceptibility to HIV-1 infection in exposed uninfected Central Africans. Retrovirology. 2006;3:35.

75. Card CM, Ball TB, Fowke KR. Immune quiescence: a model of protection against HIV infection. Retrovirology. 2013;10:141.

76. Koning FA, Otto SA, Hazenberg MD, Dekker L, Prins M, Miedema F, et al. Low-level CD4+ T cell activation is associated with low susceptibility to HIV-1 infection. J Immunol. 2005;175(9):6117-22.

77. McLaren PJ, Ball TB, Wachihi C, Jaoko W, Kelvin DJ, Danesh A, et al. HIV-exposed seronegative commercial sex workers show a quiescent phenotype in the CD4+ T cell compartment and reduced expression of HIV-dependent host factors. J Infect Dis. 2010;202 Suppl 3:S339-44.

78. Chachage M, Podola L, Clowes P, Nsojo A, Bauer A, Mgaya O, et al. Helminth-associated systemic immune activation and HIV co-receptor expression: response to albendazole/praziquantel treatment. PLoS Negl Trop Dis. 2014;8(3):e2755.

79. Kroidl I, Saathoff E, Maganga L, Makunde WH, Hoerauf A, Geldmacher C, et al. Effect of Wuchereria bancrofti infection on HIV incidence in southwest Tanzania: a prospective cohort study. Lancet. 2016;388(10054):1912-20.

80. Nielsen NO, Simonsen PE, Magnussen P, Magesa S, Friis H. Cross-sectional relationship between HIV, lymphatic filariasis and other parasitic infections in adults in coastal northeastern Tanzania. Trans R Soc Trop Med Hyg. 2006;100(6):543-50.

81. Adjobimey T, Hoerauf A. Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation. Ann Trop Med Parasitol. 2010;104(6):455-64.

82. Ludwig-Portugall I, Layland LE. TLRs, Treg, and B Cells, an Interplay of Regulation during Helminth Infection. Front Immunol. 2012;3:8.

83. Zander RA, Butler NS. Dysfunctional adaptive immunity during parasitic infections. Curr Immunol Rev. 2013;9(3):179-89.

84. Schietinger A, Greenberg PD. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. Trends Immunol. 2014;35(2):51-60.

85. Wherry EJ. T cell exhaustion. Nat Immunol. 2011;12(6):492-9.

86. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol. 2015;15(8):486-99.

87. Doering TA, Crawford A, Angelosanto JM, Paley MA, Ziegler CG, Wherry EJ. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. Immunity. 2012;37(6):1130-44.

88. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol. 2003;77(8):4911-27.

89. Buggert M, Tauriainen J, Yamamoto T, Frederiksen J, Ivarsson MA, Michaelsson J, et al. T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection. PLoS Pathog. 2014;10(7):e1004251.

90. Intlekofer AM, Takemoto N, Kao C, Banerjee A, Schambach F, Northrop JK, et al. Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. J Exp Med. 2007;204(9):2015-21.

91. Intlekofer AM, Takemoto N, Wherry EJ, Longworth SA, Northrup JT, Palanivel VR, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nat Immunol. 2005;6(12):1236-44.

92. Pearce EL, Mullen AC, Martins GA, Krawczyk CM, Hutchins AS, Zediak VP, et al. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science. 2003;302(5647):1041-3.

93. Paley MA, Kroy DC, Odorizzi PM, Johnnidis JB, Dolfi DV, Barnett BE, et al. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. Science. 2012;338(6111):1220-5.

94. Layland LE, Mages J, Loddenkemper C, Hoerauf A, Wagner H, Lang R, et al. Pronounced phenotype in activated regulatory T cells during a chronic helminth infection. J Immunol. 2010;184(2):713-24.

95. Lima NF, Gonçalves-Lopes RM, Kruize YCM, Yazdanbakhsh M, Ferreira MU. CD39 and immune regulation in a chronic helminth infection: The puzzling case of Mansonella ozzardi. PLoS Negl Trop Dis. 2018;12(3):e0006327.

96. Taylor S, Huang Y, Mallett G, Stathopoulou C, Felizardo TC, Sun MA, et al. PD-1 regulates KLRG1(+) group 2 innate lymphoid cells. J Exp Med. 2017;214(6):1663-78.

97. Cenini P, Berhe N, Hailu A, McGinnes K, Frommel D. Mononuclear cell subpopulations and cytokine levels in human visceral leishmaniasis before and after chemotherapy. J Infect Dis. 1993;168(4):986-93.

98. McKinnon KM. Flow Cytometry: An Overview. Curr Protoc Immunol. 2018;120:5.1.-5.1.11.

99. Semnani RT, Liu AY, Sabzevari H, Kubofcik J, Zhou J, Gilden JK, et al. Brugia malayi microfilariae induce cell death in human dendritic cells, inhibit their ability to make IL-12 and IL-10, and reduce their capacity to activate CD4+ T cells. J Immunol. 2003;171(4):1950-60.

100. Arndts K, Deininger S, Specht S, Klarmann U, Mand S, Adjobimey T, et al. Elevated adaptive immune responses are associated with latent infections of Wuchereria bancrofti. PLoS Negl Trop Dis. 2012;6(4):e1611.

101. Arndts K, Klarmann-Schulz U, Batsa L, Debrah AY, Epp C, Fimmers R, et al. Reductions in microfilaridermia by repeated ivermectin treatment are associated with lower Plasmodium-specific Th17 immune responses in Onchocerca volvulus-infected individuals. Parasit Vectors. 2015;8:184.

102. Arndts K, Specht S, Debrah AY, Tamarozzi F, Klarmann Schulz U, Mand S, et al. Immunoepidemiological profiling of onchocerciasis patients reveals associations with microfilaria loads and ivermectin intake on both individual and community levels. PLoS Negl Trop Dis. 2014;8(2):e2679.

103. Germann A, Oh YJ, Schmidt T, Schön U, Zimmermann H, von Briesen H. Temperature fluctuations during deep temperature cryopreservation reduce PBMC recovery, viability and T-cell function. Cryobiology. 2013;67(2):193-200.

104. Boahen O, Owusu-Agyei S, Febir LG, Tawiah C, Tawiah T, Afari S, et al. Community perception and beliefs about blood draw for clinical research in Ghana. Trans R Soc Trop Med Hyg. 2013;107(4):261-5.

105. Howie SR. Blood sample volumes in child health research: review of safe limits. Bull World Health Organ. 2011;89(1):46-53.

106. Peplow C, Assfalg R, Beyerlein A, Hasford J, Bonifacio E, Ziegler AG. Blood draws up to 3% of blood volume in clinical trials are safe in children. Acta Paediatr. 2019;108(5):940-4.

107. de Ruiter K, van Staveren S, Hilvering B, Knol E, Vrisekoop N, Koenderman L, et al. A field-applicable method for flow cytometric analysis of granulocyte activation: Cryopreservation of fixed granulocytes. Cytometry A. 2018;93(5):540-7.

108. Maecker HT, McCoy JP, Jr., Amos M, Elliott J, Gaigalas A, Wang L, et al. A model for harmonizing flow cytometry in clinical trials. Nat Immunol. 2010;11(11):975-8.

109. Silva MH, Lepzien R, Ols S, Dahlberg B, Grunewald J, Loré K, et al. Stabilization of blood for long-term storage can affect antibody-based recognition of cell surface markers. J Immunol Methods. 2020;481-482:112792.

110. Pinto LA, Trivett MT, Wallace D, Higgins J, Baseler M, Terabe M, et al. Fixation and cryopreservation of whole blood and isolated mononuclear cells: Influence of different procedures on lymphocyte subset analysis by flow cytometry. Cytometry B Clin Cytom. 2005;63(1):47-55.

111. Horton J, Klarmann-Schulz U, Stephens M, Budge PJ, Coulibaly Y, Debrah A, et al. The design and development of a multicentric protocol to investigate the impact of adjunctive doxycycline on the management of peripheral lymphoedema caused by lymphatic filariasis and podoconiosis. Parasit Vectors. 2020;13(1):155.

112. Ahmed MIM, Ntinginya NE, Kibiki G, Mtafya BA, Semvua H, Mpagama S, et al. Phenotypic Changes on Mycobacterium Tuberculosis-Specific CD4 T Cells as Surrogate Markers for Tuberculosis Treatment Efficacy. Frontiers in immunology. 2018;9:2247-.

113. Lovelace P, Maecker HT. Multiparameter intracellular cytokine staining. Methods in molecular biology (Clifton, NJ). 2011;699:165-78.

114. Maecker HT, Dunn HS, Suni MA, Khatamzas E, Pitcher CJ, Bunde T, et al. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. J Immunol Methods. 2001;255(1-2):27-40.

115. Riedhammer C, Halbritter D, Weissert R. Peripheral Blood Mononuclear Cells: Isolation, Freezing, Thawing, and Culture. Methods Mol Biol. 2016;1304:53-61.

116. Paredes RM, Tadaki DK, Sooter A, Gamboni F, Sheppard F. Cryopreservation of human whole blood allows immunophenotyping by flow cytometry up to 30days after cell isolation. J Immunol Methods. 2018;452:32-8.

117. Mazur P. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. Cryobiology. 1977;14(3):251-72.

118. Kohlmeier JE, Miller SC, Smith J, Lu B, Gerard C, Cookenham T, et al. The chemokine receptor CCR5 plays a key role in the early memory CD8+ T cell response to respiratory virus infections. Immunity. 2008;29(1):101-13.

119. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. Trends Immunol. 2015;36(4):265-76.

120. Jia B, Zhao C, Rakszawski KL, Claxton DF, Ehmann WC, Rybka WB, et al. Eomes(+)T-bet(low) CD8(+) T Cells Are Functionally Impaired and Are Associated with Poor Clinical Outcome in Patients with Acute Myeloid Leukemia. Cancer Res. 2019;79(7):1635-45.

121. Pauken KE, Wherry EJ. SnapShot: T Cell Exhaustion. Cell. 2015;163(4):1038-.e1.

122. Osuch S, Laskus T, Berak H, Perlejewski K, Metzner KJ, Paciorek M, et al. Decrease of T-cells exhaustion markers programmed cell death-1 and T-cell immunoglobulin and mucin domain-containing protein 3 and plasma IL-10 levels after successful treatment of chronic hepatitis C. Sci Rep. 2020;10(1):16060.

123. Shenoy RK. Clinical and pathological aspects of filarial lymphedema and its management. Korean J Parasitol. 2008;46(3):119-25.

124. Lovelace P, Maecker HT. Multiparameter intracellular cytokine staining. Methods Mol Biol. 2011;699:165-78.

125. Ritter M, Ndongmo WPC, Njouendou AJ, Nghochuzie NN, Nchang LC, Tayong DB, et al. Mansonella perstans microfilaremic individuals are characterized by enhanced type 2 helper T and regulatory T and B cell subsets and dampened systemic innate and adaptive immune responses. PLoS Negl Trop Dis. 2018;12(1):e0006184.

126. Ritter M, Osei-Mensah J, Debrah LB, Kwarteng A, Mubarik Y, Debrah AY, et al. Wuchereria bancrofti-infected individuals harbor distinct IL-10-producing regulatory B and T cell subsets which are affected by anti-filarial treatment. PLoS Negl Trop Dis. 2019;13(5):e0007436.

127. Aravindhan V, Anand G. Cell Type-Specific Immunomodulation Induced by Helminthes: Effect on Metainflammation, Insulin Resistance and Type-2 Diabetes. Am J Trop Med Hyg. 2017;97(6):1650-61.

128. Ayelign B, Akalu Y, Teferi B, Molla MD, Shibabaw T. Helminth Induced Immunoregulation and Novel Therapeutic Avenue of Allergy. J Asthma Allergy. 2020;13:439-51.

129. Moyat M, Coakley G, Harris NL. The interplay of type 2 immunity, helminth infection and the microbiota in regulating metabolism. Clin Transl Immunology. 2019;8(11):e01089.

130. Murthy PK. Strategies to Control Human Lymphatic Filarial Infection: Tweaking Host's Immune System. Curr Top Med Chem. 2019;19(14):1226-40.

131. Satapathy AK, Sartono E, Sahoo PK, Dentener MA, Michael E, Yazdanbakhsh M, et al. Human bancroftian filariasis: immunological markers of morbidity and infection. Microbes Infect. 2006;8(9-10):2414-23.

132. Kroidl I, Chachage M, Mnkai J, Nsojo A, Berninghoff M, Verweij JJ, et al. Wuchereria bancrofti infection is linked to systemic activation of CD4 and CD8 T cells. PLoS Negl Trop Dis. 2019;13(8):e0007623.

133. Babu S, Nutman TB. Proinflammatory cytokines dominate the early immune response to filarial parasites. J Immunol. 2003;171(12):6723-32.

134. Olszewski WL, Jamal S, Manokaran G, Pani S, Kumaraswami V, Kubicka U, et al. Bacteriologic studies of skin, tissue fluid, lymph, and lymph nodes in patients with filarial lymphedema. Am J Trop Med Hyg. 1997;57(1):7-15.

135. Shenoy RK, Kumaraswami V, Suma TK, Rajan K, Radhakuttyamma G. A double-blind, placebo-controlled study of the efficacy of oral penicillin, diethylcarbamazine or local treatment of the affected limb in preventing acute adenolymphangitis in lymphoedema caused by brugian filariasis. Ann Trop Med Parasitol. 1999;93(4):367-77.

136. Zuiqar H, Malik A. Bancroftian Filariasis. StatPearls. Treasure Island (FL): StatPearls Publishing

137. Lederman MM, Penn-Nicholson A, Cho M, Mosier D. Biology of CCR5 and its role in HIV infection and treatment. Jama. 2006;296(7):815-26.

138. Lopalco L. CCR5: From Natural Resistance to a New Anti-HIV Strategy. Viruses. 2010;2(2):574-600.

139. Ellwanger JH, Kaminski VL, Rodrigues AG, Kulmann-Leal B, Chies JAB. CCR5 and CCR5Δ32 in bacterial and parasitic infections: Thinking chemokine receptors outside the HIV box. Int J Immunogenet. 2020;47(3):261-85.

140. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol. 2009;10(1):29-37.

141. Du H, Yi Z, Wang L, Li Z, Niu B, Ren G. The co-expression characteristics of LAG3 and PD-1 on the T cells of patients with breast cancer reveal a new therapeutic strategy. Int Immunopharmacol. 2020;78:106113.

142. Mishra AK, Kadoishi T, Wang X, Driver E, Chen Z, Wang XJ, et al. Squamous cell carcinomas escape immune surveillance via inducing chronic activation and exhaustion of CD8+ T Cells co-expressing PD-1 and LAG-3 inhibitory receptors. Oncotarget. 2016;7(49):81341-56.

143. Golden-Mason L, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, McMahon BJ, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. J Virol. 2009;83(18):9122-30.

144. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, et al. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J Exp Med. 2008;205(12):2763-79.

145. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. Immunology. 2010;129(4):474-81.

146. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. J Immunol. 2006;176(5):3248-56.

147. Rajamanickam A, Munisankar S, Dolla C, Nutman TB, Babu S. Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4)- and Programmed Death 1 (PD-1)-Mediated Regulation of Monofunctional and Dual Functional CD4(+) and CD8(+) T-Cell Responses in a Chronic Helminth Infection. Infect Immun. 2019;87(12).

148. Maizels RM, Bundy DA, Selkirk ME, Smith DF, Anderson RM. Immunological modulation and evasion by helminth parasites in human populations. Nature. 1993;365(6449):797-805.

Appendix A:

	Ghana	Stage 1-2	Stage 3-4	Stage 5-7	**Tanzania**	Stage 1-2	Stage 3-4	Stage 5-7
Sample size (n)	60	25	15	20	114	32	46	36
Mean age [range]	48.1 [19-65]	47.8 [19-65]	48.0 [19-65]	48.0 [19-65]	48.8 [15-65]	48.8 [15-65]	48.7 [15-65]	49.1 [15-65]
Gender (Female:Male) [%]	53:7 [88:12]	21:4 [84:16]	15:0 [100:0]	17:3 [85:15]	69:45 [61:39]	22:10 [69:31]	29:17 [63:37]	18:18 [50:50]
Mean years living in the endemic area [range]	47.7 [19-65]	47.4 [19-65]	47.6 [19-65]	47.6 [19-65]	42.0 [3-65]	42.0 [3-65]	42.3 [3-65]	42.2 [3-65]
Median MDA rounds [range]	5 [1-15]	5 [1-13]	5 [1-13]	5 [1-15]	4 [0-15]	4 [0-15]	4 [0-15]	4 [0-15]
Median lymphedema stage [SD]	3 [1.8]	NA	NA	NA	3 [1.7]	NA	NA	NA

Table 1: Participants characteristics per leg lymphedema stage in the LEDoxy clinical trial in Ghana and Tanzania.

	EN	Wb-inf.	LE
Sample size (n)	58	29	37
Mean age [range]	45.1 [21-81]	44.2 [20-83]	48.4 [26-64]
Gender (Female:Male) [%]	39:19 [67:33]	17:12 [59:41]	30:7 [81:19]
Mean years living in the endemic area [range]	39.2 [6-81]	42.8 [20-83]	48.4 [26-64]
Median MDA rounds [range]	2 [0-6]	4 [1-8]	6 [1-15]
Median lymphedema stage [SD]	NA	NA	3 [1.7]

Table 2: Ghanaian participant characteristics for the CD4+ T cell activation and CD8+ T cell exhaustion data.

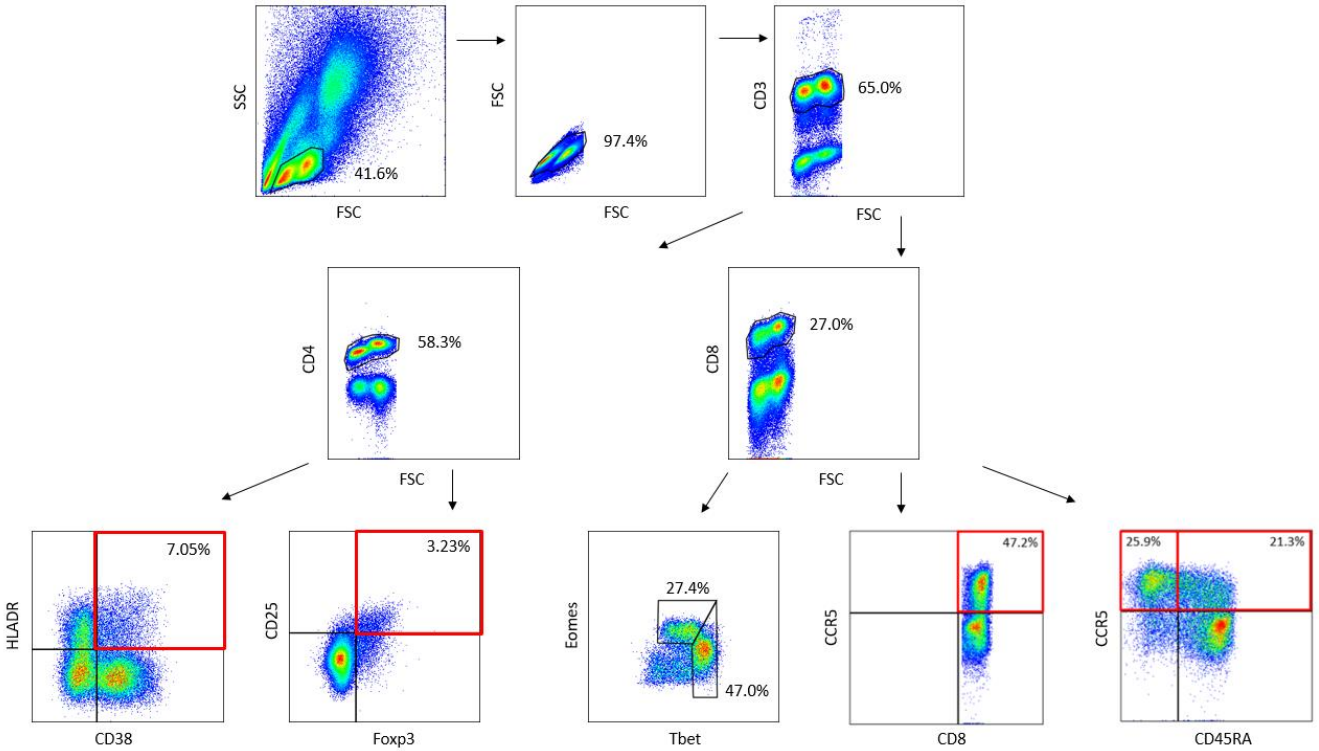
Appendix B:

Location	Protein Target	Clone	Fluorophore	Company
Extracellular molecules	b7	FIB27	PE	Biolegend
	CD3	UCHT1	ECD	Beckman Coulter
	CD4	OKT4	PerCP-Cy5.5	Invitrogen
	HLA-DR	LN3	PeCy7	Invitrogen
	CD38	HIT2	APC	Biolegend
	CD195 (CCR5)	REA245	APC	Miltenyi Biotech
	CD27	M-T271	APC-H7	BD Biosciences
	CD45RA	HI100	Brilliant Violet 421	Biolegend
	CD8	RPA-T8	V500	BD Biosciences
	CD25	BC96	Brilliant Violet 605	Biolegend
Intracellular molecules	FoxP3	259D	Alexa Fluor 488	Biolegend
	Tbet	eBio4B10	PE	eBioscience
	Eomes	WD1928	PeCy7	Invitrogen

Table 3: Whole blood flow cytometry panel information.

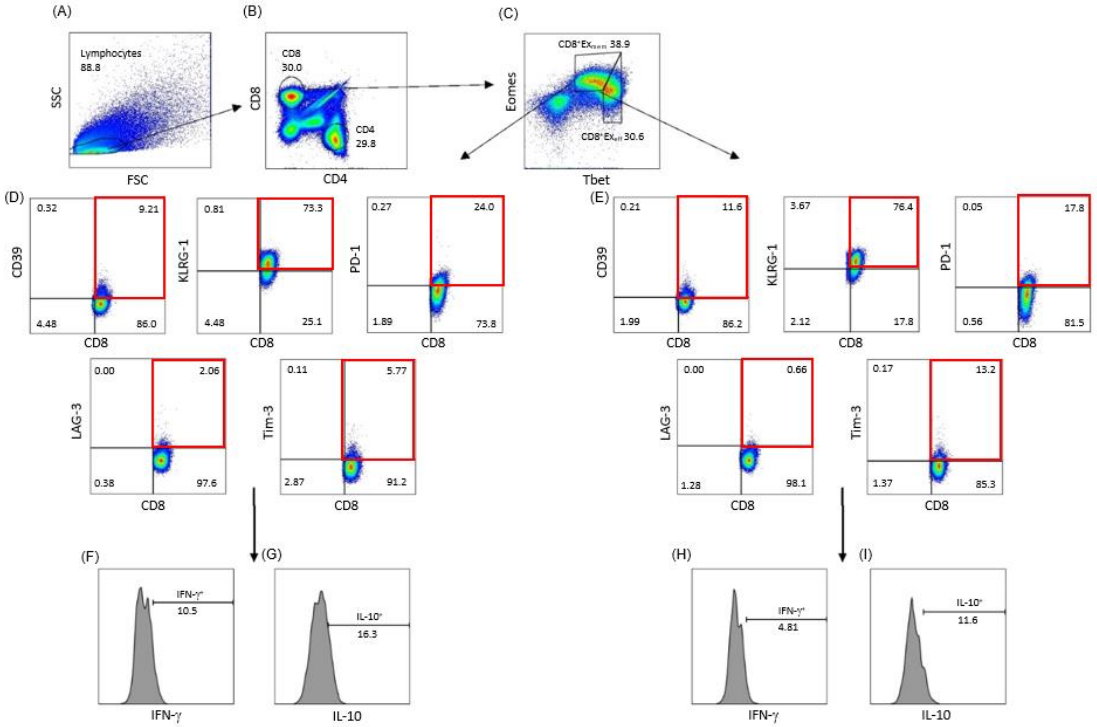
APC: allophycocyanin; APC-H7: allophycocyanin H7; ECD: R phycoerythrin-Texas Red-X; PE: phycoerythrin; PECy7: phycoerythrin Cy7; PerCP-Cy5.5: peridinin chlorophyll protein; V500: violet 500

Appendix C:



Whole blood sample complete gating strategy.

Appendix D:



PBMC sample complete gating strategy.

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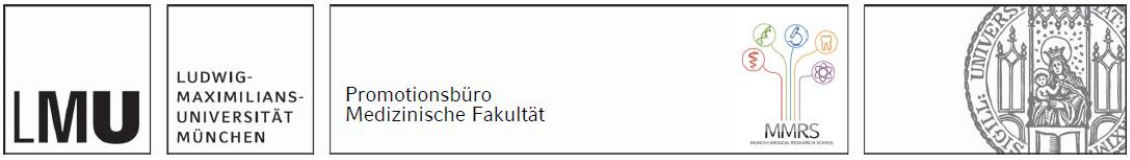
Affidavit



Affidavit

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I hereby declare, that the submitted thesis entitled:

Investigation of filarial associated immune modulation and effects on HIV susceptibility

is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 09.12.2021
place, date

Sacha Horn
Signature doctoral candidate

Confirmation of congruency

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Confirmation of congruency between printed and electronic version of the doctoral thesis

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I hereby declare, that the submitted thesis entitled:

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is congruent with the printed version both in content and format.

Munich, 09.12.2021
place, date

Sacha Horn
Signature doctoral candidate

List of publications

Published manuscripts

Horn S, Ahmed MIM, Geldmacher C, Marandu TF, Osei-Mensah J, Debrah A, Layland LE, Hoerauf A, Kroidl I. Flow cytometric analysis of cell lineage and immune activation markers using minimal amounts of human whole blood-Field method for remote settings. J Immunol Methods. 2021 Apr;491:112989. doi: 10.1016/j.jim.2021.112989. Epub 2021 Feb 9. PMID: 33571509.

Submitted manuscripts

Sacha Horn, Dennis Borrero-Wolff, Manuel Ritter, Kathrin Arndts, Anna Wiszniewsky, Linda Batsa Debrah, Alexander Y. Debrah, Jubin Osei-Mensah, Mkunde Chachage, Achim Hoerauf, Inge Kroidl, Laura E. Layland**. "**Distinct immune profiles of exhausted effector and memory $CD8^+$ T cells in individuals with filarial lymphedema"

Under review in Frontier Immunology

Inge Kroidl, Mohamed I.M. Ahmed, **Sacha Horn**, Christina Polyak, Allahna Esber, Ajay Parikh, Leigh Anne Eller, Hannah Kibuuka, Michael Semwogerere, Betty Mwesigwa, Prossy Naluyima, Joy Mary Kasumba, Jonah Maswai, John Owuoth, Valentine Singóei, Eric Rono, Rebecca Loose, Michael Hoelscher, Julie A Ake, Christof Geldmacher. "Assessment of tuberculosis disease activity in mycobacterium tuberculosis infected people living with HIV"

Under review in the American Journal of Respiratory and Critical Care Medicine

Manuscripts in preparation

Basel Habboub, Mkunde Chachage, Lucas Maganga, Petra Clowes, **Sacha Horn**, Nyanda Ntinginya, Leonard Maboko, Michael Hoelscher, Elmar Saathoff,

Inge Kroidl. “Exploring the novel association between bed net ownership and HIV risk in adults; data from the EMINI cohort in Mbeya, Tanzania 2006-2011”

Jonathan Mnkai, Thomas Marandu, Jacklina Mhidze, Agatha Urio, Lucas Maganga, Antelmo Haule, Nhamo Chiwerengo, **Sacha Horn**, Maureen Mosoba, Wilfred Lazarus, Alex Debrah, Achim Hoerauf, Michael Hoelscher, Friedrich Rieß, Elmar Saathof, Mkunde Chachage, Inge Kroidl. “Step towards elimination of *W. bancrofti* in Southwest-Tanzania 10 years after mass drug administration with Albendazole/Ivermectin”