Lymphatic Filariasis: Host and Parasite Factors and the Pathogenesis of Systemic Adverse Events Following Treatment

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Lymphatic Filariasis: Host and Parasite Factors and the Pathogenesis of Systemic Adverse Events Following Treatment

by

Britt Juul Andersen

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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ABSTRACT OF THE DISSERTATION

Lymphatic Filariasis: Host and Parasite Factors and the Pathogenesis of Systemic Adverse Events Following Treatment

by

Britt Juul Andersen

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Microbiology and Microbial Pathogenesis

Washington University in St. Louis, 2020

Professor Gary J. Weil, Chair

Lymphatic filariasis (LF) is a neglected tropical disease caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. The primary tool used by the Global Program to Eliminate LF is mass drug administration (MDA), and some 500 million people take the medications each year. Mild to moderate adverse events (AEs) are common after LF treatment, and these pose a major challenge for the LF elimination program. To better understand the pathogenesis of AEs, we studied patients from LF treatment trials in Côte d’Ivoire and Papua New Guinea, where plasma and leucocytes were collected pre and post-treatment and subjects were monitored for AEs. We found that plasma levels of filarial antigen and DNA increased post-treatment in individuals with AEs. We discovered that a whole range of filarial antigens with the AD12 epitope circulate in the bloodstream 24 hours after treatment, in contrasts to the widely accepted notion that the high molecular weight Circulating Filarial Antigen (CFA) is the only antigen present the blood of *W. bancrofti*-infected individuals. We then investigated the
cytokine response during AEs by measuring 27 cytokines pre and post-treatment. We identified 11-16 cytokines that increased post-treatment in individuals with AEs. This complex cytokine response could be consistent with a LPS-like response (caused by exposure to Wolbachia lipoprotein) with increases in TNF-α, IL-1β, IL-6, IL-1RA and IL-10.

To further delineate the host response during AEs, a transcriptomic analysis was completed. Global RNA sequencing was performed for 9 individuals with systemic AEs and for 9 matched controls without AEs. Differential gene expression analysis identified a significant transcriptional signature associated with post-treatment AEs; 744 genes were significantly up-regulated in the AE group (post versus pre-treatment, paired). These genes were enriched for many biological pathways, including pro-inflammatory pathways such as TLR and NF-kappa B signaling. A machine-learning tool was used to prioritize the genes up-regulated post-treatment in individuals with AEs, in order to identify the genes that had the best correlation between expression levels and AE classification, and in order to identify a subset of genes to validate with RT-qPCR. Increased expression of seven out of the top eight genes identified were validated with RT-qPCR. TLR2 was identified by the machine-learning tool to be highly correlated to the development of AEs, and this gene was confirmed to increase post-treatment in individuals with AEs by RT-qPCR. These results suggest that Wolbachia lipoprotein is involved in AE development because it is known to signal though TLR2-TLR6 and activate downstream NF-kappa B. Additional support for this hypothesis was the discovery that LPS Binding Protein (LBP) increased post-treatment in individuals with AEs, because LBP can shuttle lipoproteins to TLR2. Improved understanding of the pathogenesis of AEs may lead to improved management or prevention that could increase MDA compliance and hasten LF elimination.
Chapter 1:

Introduction to lymphatic filariasis
Preface

This chapter was written by BJA. Comments from GJW and PUF were incorporated into the final version, presented here. Please note that this thesis and associated literature review was completed by April 2018.
1.1 **Introduction to lymphatic filariae.**

1.1.1 **Overview.**

Lymphatic filariasis (LF) is an important neglected tropical disease that is caused by threadlike nematode parasites that live in the lymphatic system of the human host. Three different lymphatic filarial species infect humans: *Wuchereria bancrofti*, *Brugia malayi* and *B. timori* (1). *W. bancrofti* is the most widespread and it is responsible for over 90% of human LF infections, whereas *B. malayi* and *B. timori* are limited to specific regions in Asia (2). In the year 2000 over 120 million people were infected with one of the three LF-causing parasites, and about 40 million of these individuals were disfigured and disabled by the disease (3). It is estimated that 856 million individuals in 52 countries across the world are currently threatened by LF and require preventative chemotherapy or treatment in order to stop the spread of the infection (3).

1.1.2 **Historical background.**

Lymphatic filariasis is an ancient disease that was first described by Hindu and Persian doctors around 600 B.C. (4). However, illustrations from as early as 1,500 B.C. that possibly portray individuals suffering from elephantiasis have been found in Egypt (Figure 1.1A) (4). Additionally, sculptures from around 500 B.C. from the Nok civilization in West Africa may show scrotal swelling (hydroceles), another characteristic of LF (Figure 1.1B) (5). The first definitive reports on LF are from the 16th century, when the Dutch explorer Jan Huygen Linschoten (1563-1611) visited Goa, and reported that the natives were “all born with one of their legs and one foot from the knee downwards as thick as an elephants leg” (6).
The first scientific discoveries regarding filariasis occurred in the 19th century when the larval form of the parasite (microfilariae or Mf) were discovered in hydrocele fluid from a Cuban man in 1863 (7), and in the blood of an infected individual in 1872 (8). Joseph Bancroft discovered the first adult female worm in a lymph node ulcer in 1877 (4), and this filarial species was later named after him, and we now know it as *W. bancrofti*. The adult male parasite was first discovered in 1888 by Sibthorpe (9). The most prominent discovery in early filariasis research was made by the father of tropical medicine, Sir Patrick Manson, in 1877. He found Mf in the stomach of bloodsucking mosquitos and thereby identified the vector of the parasite, and helped elucidate the parasite life cycle (10). This discovery is considered the birth of medical entomology, and as one of the most significant discoveries in the field of tropical medicine, with implications that went beyond helminthology into research areas such as malaria and arboviruses.

Despite the advancement in the field of LF research, treatment for the disease was not available until the mid 20th century when it was discovered that diethylcarbamazine (DEC) showed remarkable effects in the treatment of *Litomosoides carinii* (now *L. sigmodontis*, a filarial nematode that infects cotton rats) and *Dirofilaria immitis* (a filarial nematode that infects dogs), and was able to clear *W. bancrofti* Mf from the blood of infected humans (11-13). Ivermectin (IMV) was introduced in 1982 as a treatment for onchocerciasis (14), and the use of this drug was expanded for the use in *W. bancrofti* infected individuals just a few years later (15). In 1995 it was discovered that co-administration of both DEC and IVM together was significantly more effective than either drug on its own (16). Finally, in 1997-1998 the third main anti-filarial drug,
albendazole (ALB), was discovered to increase the efficacy of DEC and IVM when added to either drug, and administered as a two-drug combination treatment (17-19). In 2000 the World Health Organization (WHO) launched the Global Program to Eliminate LF (GPELF) (20).

1.1.3 Life cycle.

The *W. bancrofti* lifecycle is depicted in Figure 1.2, and it is representative of all the LF-causing filarial nematodes, as their lifecycles are very similar. The LF-causing filarial nematodes are vector-born, and are transmitted by mosquitoes from a number of genera (including *Culex, Anopheles, Mansonia, Ochlerotatus, Coquillettidia* and *Aedes*) depending on the geographical location (21). The 4-8 cm long adult parasites live in the lymphatic vessels of the human host, and the females release first stage larvae (L1) commonly called microfilariae (Mf) into the blood (1). A single adult female worm can release up to 10,000 Mf per day (22), and they measure approximately 260 x 8 um (1). The lifespan of Mf is thought to be around one year (23). The Mf circulate in the blood of infected individuals, where they have an interesting periodicity. *W. bancrofti* Mf are usually most abundant in the circulation during the night (nocturnal periodicity) when the vector mosquito typically takes blood meals from the host, whereas they sequester in the deep vascular beds during the day (1). The Mf are ingested by female mosquitoes when the mosquito feeds on an infected human host. The Mf then migrate to the thoracic muscle of the mosquito, where they undergo two molts (from L1 to L2 and from L2 to L3) to become the infective third-stage larvae (L3). The larval development in the mosquito takes a minimum of 10-12 days. Mature L3 migrate to the mouthparts of the
mosquito where they can initiate new infections when they are transferred to humans with the mosquito’s next blood meal. Infective larvae migrate to lymphatic vessels in the human and grow to develop into mature adult worms over a period of several months. If both male and female worms parasitize the same host they will reproduce, and the females will start producing Mf, and the cycle can continue (1). The reproductive life span of lymphatic filarial parasites is an average 4 to 6 years.

1.1.4 Wolbachia endobacteria of filarial nematodes.

Wolbachia are intracellular endosymbiotic bacteria that infect insects and nematodes. The Wolbachia genus belongs to the family Anaplasmataceae and the order Rickettsiales (alphaproteobacteria). There is a single species in the Wolbachia genus: Wolbachia pipiens (24). Wolbachia was discovered to be an essential endosymbiont of most filarial worms in 1999 (25). Many filarial nematodes, such as the lymphatic filariae (W. bancrofti and the Brugia species) (26) and Onchocerca volvulus (causes onchocerciasis) (27) are dependent on the Wolbachia endosymbiont for development, fertility and survival, and the bacteria are maternally inherited (28). However, Wolbachia is absent from Loa loa (29), the rodent filaria Acanthocheilonema viteae (30), the deer parasite Onchocerca flexuosa (31) and many other species that infect wildlife (32). In W. bancrofti and the brugian species of filarial nematodes, Wolbachia are present in all developmental stages, with low amounts in the vector stages and increased amounts in the mammalian stages (30, 33-35). In adult worms Wolbachia localizes near the reproductive system and lateral cords, whereas it is absent from the nervous system, digestive system and the muscles (Figure 1.3).
1.2 Lymphatic filariasis disease and treatment.

1.2.1 Filarial disease manifestations.

LF and the host’s inflammatory response to the parasite can lead to severe morbidity and disability with lymphedema, hydrocele, and elephantiasis (Figure 1.4). LF is considered to be the second most common cause of long-term disability worldwide after mental illness (19, 36). The clinical manifestations of LF range from subclinical to pronounced chronic disease causing severe disability. Most individuals infected with LF-causing parasites have no clinical symptoms, even though Mf are present in their blood (37). Acute manifestations of LF include acute filarial lymphangitis (AFL) and acute dermatolymphangioadenitis (ADLA). AFL is thought to be induced by the death of adult parasites, and it presents as acute inflammation of a lymphatic vessel. ADLA, on the other hand, is caused by a secondary bacterial infection and is often associated with a history of injury to the skin. ADLA typically causes severe pain, fever and chills (38, 39).

Recurrent acute attacks represent major risk factors for the development of chronic disease manifestations. It was estimated that approximately 40 million individuals live with symptoms of chronic LF, and this includes an estimated 25 million men with hydroceles and an estimated 15 million individuals with lymphedema or elephantiasis (22). Hydroceles (Figure 1.4A) are the most common chronic presentations of LF, and they are caused by the accumulation of lymph fluid in the tunica vaginalis of the scrotal sac, in addition to possible thickening of the spermatic cord and changes in the skin and subcutaneous tissue around the scrotum (40). Filarial hydroceles can have a severe negative impact on health and result in stigmatization of the affected men (41-43). Chronic lymphedema is another common manifestation of filarial disease, and it typically
affects the lower legs (Figure 1.4B). Adult parasites reside in the lymphatic system and can therefore cause damage to this system. Although live worms do not induce strong inflammatory responses, granulomatous reactions occur around dying worms (44, 45), and this can lead to lymphatic damage. Over time the edema worsens, and it can progress to elephantiasis (Figure 1.4C/D), which is characterized by deep skin folds and skin fissuring that can provide pathways for entry of microorganisms (46). These secondary bacterial and fungal infections can in turn accelerate disease progression and worsen symptoms. Lymphedema and elephantiasis also have severe negative impacts on the afflicted individuals and their caretakers (47).

1.2.2 Immune response during lymphatic filariasis infection.

Helminths differ from other types of pathogens because they are multicellular, typically do not reproduce within the host, are highly motile, and often parasitize the host for many years. Helminth infections therefore induce distinct types of immune responses. Other microorganisms such as bacteria and viruses normally elicit a pro-inflammatory Th1 immune response, whereas the anti-inflammatory Th2 response usually dominates in helminth infections. The typical host immune response seen in human filarial infections involves the production of cytokines: IL-4, IL-5, IL-9, IL-10 and IL-13; the antibody isotypes IgG1, IgG4 and IgE; and expanded populations of eosinophils and immunoregulatory monocytes (48). One study concluded that filarial infections are associated with decreased parasite specific Th1 responses, such as decreased IL-2 and IFN-γ, but normal or increased levels of parasite antigen-specific Th2 cytokines, such as IL-4, IL-5 and IL-10 (49). Various studies have compared the cytokine profiles in the
following clinical groups of filariasis patients: asymptomatic filarial-infected patients (INF), individuals with chronic lymphatic obstruction (CP), and endemic normals with no circulating Mf (EN). In a study from 1997, peripheral blood mononuclear cells (PBMCs) were isolated from patients in the different clinical categories, and stimulated with filarial antigens (50). This study concluded that cytokine profiles of PBMCs were different between these categorized clinical groups, with INF PBMCs expressing elevated Th2 type cytokines like IL-4, IL-5 and IL-10 and decreased levels of IL-2 and IFN-γ in response to parasite antigen, whereas CP individuals produced elevated levels of both Th1 and Th2 type cytokines in response to parasite antigen. It is hypothesized that pro-inflammatory immune responses are responsible for the symptoms observed in CP patients. However, most individuals infected with LF are asymptomatic, and it is believed that a Th2 dominant response allows the immune system to tolerate the infection without establishing a symptom-producing pro-inflammatory environment. In a review from 2001, Allen et al. suggests the following scenario: Live filarial parasites secrete immunomodulatory molecules that induce Th2 responses, whereas dead parasites release different substances for example from intracellular Wolbachia, leading to the activation of macrophages with the production of pro-inflammatory cytokines and a Th1 type response. As more parasites die the balance shifts from a Th2 response to a Th1 response producing the symptoms of chronic filarial infections (51). Using this model to consider what happens after anti-filarial treatment one could hypothesize that microfilaricidal drugs, such as IVM and DEC, would result in an immediate shift from a Th2 to a Th1 response due to the huge number of dead or dying Mf.
1.2.3 Treatment of lymphatic filariasis.

The World Health Organization (WHO) launched the Global Program to Eliminate LF (GPELF) in 2000 with the goal of eliminating LF as a public health problem by 2020 (2). To reach this goal, mass drug administration (MDA) programs have been implemented in 61 countries across the world, and approximately 6 billion doses of anti-filarial medications have been distributed, making GPELF the largest public health intervention to date based on MDA (Figure 1.5) (3, 52). Historically, LF was treated with a 12-day course of DEC (19). The current treatments used in LF MDA programs include annual distribution of two-drug regimens: IVM (150 µg/kg) plus ALB (400 mg) in areas co-endemic for onchocerciasis, or DEC (6 mg/kg) plus ALB (400 mg) in the rest of the world. Exceptions to the two-drug regimens include areas that are co-endemic for *L. loa* where only ALB can be safely used (400 mg. ALB preferably twice a year), and the new triple therapy (“IDA”, discussed below). LF MDA programs have been successful in decreasing LF infection parameters such as microfilaremia (Mf present in the blood), filarial antigenemia and anti-filarial antibody rates in treated populations (53). The implementation of MDA is based on community diagnosis to identify endemic areas followed by distribution of anti-filarial medications annually for 5-8 years to entire at-risk populations. There is a clear correlation between the number of completed treatment rounds and infection clearance (54). The GPELF strategy has been shown to be feasible and cost-effective (55, 56), in large part because the drugs are donated by pharmaceutical partners.

A new and exciting breakthrough in the field of LF treatment was the discovery that co-administration of all three anti-filarial drugs, IVM, DEC and ALB (sometimes
referred to as IDA), had increased efficacy against LF. The first study was conducted in Papua New Guinea and published in 2016 (57). In this pilot study the efficacy, safety, and pharmacokinetics of single-dose IVM, DEC and ALB was tested in *W. bancrofti*-infected adults. Twelve individuals were treated with the new triple therapy and twelve individuals were treated with the standard LF treatment for Papua New Guinea (DEC plus ALB). The results were surprisingly impressive as all 12 individuals who received the triple therapy were Mf negative one year post-treatment, whereas only 1 of 12 individuals treated with standard double therapy were Mf negative at one year post-treatment (57). Larger clinical trials to test the efficacy of the triple therapy are underway in Papua New Guinea (Clinicaltrials.gov NCT # 01975441) and Côte d'Ivoire (Clinicaltrials.gov NCT # 02974049), and the new data appears to confirm the finding that IDA is more effective than the standard LF treatment regimens (58, 59). The safety of the triple therapy treatment is currently being evaluated in large community-based studies in India, Indonesia, Haiti, Papua New Guinea and Fiji, where over 10,000 individuals have been treated with IDA (ClinicalTrials.gov Identifier: NCT02899936). Based on this extensive safety data, the WHO recently recommended IDA as the preferred treatment for certain LF endemic areas (60).

1.2.4 Mechanism of action of the anti-filarial drugs.

ALB binds to nematode tubulin thereby inhibiting polymerization and the assembly into microtubules. This causes degenerative changes in the tegument and intestinal cells of the parasite, which results in impaired uptake of glucose, and the glycogen stores in the parasite are depleted (61). The degenerative alterations also result
in decreased production of adenosine triphosphate by the parasite, which is an important source of energy required for survival. Ultimately the parasite is immobilized because of this diminished energy production, and it eventually dies.

DEC is an antifilarial drug that is structurally dissimilar from ALB and IVM, and it has been used in the treatment of LF since 1947. DEC inhibits arachidonic acid metabolism and inducible nitric oxide synthase (62). The mode of action by which DEC kills LF parasites is still poorly understood. It is particularly remarkable that DEC exerts its anti-filarial effects within minutes *in vivo* with decreasing Mf counts, but it has virtually no effect on Mf *in vitro* (63). This indicates that DEC requires host factors for its activity, and previous work has suggested that the innate immune system is involved (64). DEC decreases circulating Mf within days of treatment, and is also believed to have some macrofilaricidal effect (65).

IVM is an avermectin compound that was initially derived from the bacterium *Streptomyces avermitilis* (66). Similar to DEC, IVM has little effect on filarial parasites *in vitro* (67), and it has been shown to interact with the host immune system (68). The mode of action by which IVM kills Mf is not completely delineated, but it interferes with glutamate-gated ion channels that can affect parasite contractility and release of immunomodulatory molecules (68). The parasite’s nervous system and muscular system is also affected by IVM.
1.3 Adverse events after anti-filarial treatment.

1.3.1 Overview and impact of adverse events.

The medications used for LF MDA; ALB, IVM and DEC, have well-established safety profiles, and severe adverse events are extremely rare (69-72). However, mild to moderate adverse events (AEs), such as fever, myalgia, headache and hypotension, are common in LF-infected individuals (2, 61, 71). The fear of AEs in communities receiving MDA is a main factor that reduces compliance (73, 74). Minimizing the impact of AEs has therefore been identified as a key component for successful MDA programs (73). Prior studies have shown that AEs are almost exclusively observed in people with microfilaremia, and the severity of AEs is strongly correlated with Mf counts (75, 76). Uninfected individuals very rarely experience AEs after LF MDA (77), so the AEs experienced by LF-infected individuals are not a result of direct drug toxicity. These observations have led to the hypothesis that AEs are caused by immune responses to dead or dying Mf and adult worms.

In contrary to the mild and moderate AEs observed when DEC and IVM are administered to LF-infected individuals, both of these drugs can cause severe AEs including encephalopathy and death when used in individuals infected with L. loa, that can exhibit very high Mf levels (78, 79). Additionally, DEC can result in loss of vision and other severe eye damage if administered to individuals infected with O. volvulus (80, 81). DEC is therefore not used in areas in Africa where LF is co-endemic with O. volvulus or L. loa. IVM is not used in areas that are co-endemic for L. loa, unless it is meso or hyperendemic for O. volvulus in which case the risk of blindness caused by onchocerciasis outweighs the risk of AEs caused by IVM treatment.
1.3.2 Host responses and adverse events following treatment of LF.

Only a few studies have examined cytokine changes following anti-filarial treatment, and the majority of these studies are older and therefore used the then recommended, but now outdated, treatment for LF, a multiday course of DEC. IL-5 has been shown to increase in sera from patients infected with *W. bancrofti* immediately following DEC treatment (82, 83) leading to a transient decrease in peripheral eosinophils as they migrate to the tissue, followed by peripheral eosinophilia at 4-6 days post-treatment (82). IL-5 has not been linked to AEs following treatment. However, other cytokines have been shown to increase after anti-filarial treatment, and have been implicated in the development of AEs. A study from 1994 collected plasma from 10 *W. bancrofti*-infected men immediately before and 2 and 6 hours after the first dose of a multiday DEC treatment, and measured IL-6 and TNF-α levels in the plasma. They found both IL-6 and TNF-α were increased in plasma collected from the 5 individuals with AEs post-treatment (84). Another study also found a strong correlation between inflammatory mediators and the severity of AEs following treatment. In this study from 2000 they used ELISAs to measure various cytokine levels before and after a 12-day treatment with daily doses of DEC in 29 *B. malayi* infected individuals. They found that increased post-treatment levels of IL-6, lipopolysaccharide binding protein (LBP), IL-10 and soluble TNF receptors (sTNF-R) were correlated with systemic reactions after DEC treatment of filarial patients, with the strongest association being with IL-6 and LBP. In this study post-treatment blood was collected at 2, 4, 8, 24, 32, 48 and 120 hours after the initial DEC dose, and IL-6 levels peaked between 8-24 hours whereas LBP levels peaked
between 24-48 hours (85). AEs after anti-filarial treatment peak at around 24-48 hours post-treatment (75, 86-89). In agreement with these results, another study concluded that IL-6, but not TNF-α was correlated with AEs, and peaked at 24 hours post-treatment; however, only four *B. malayi* infected individuals were included in that study (87). A single published study found no correlation between IL-6 and AEs following a single dose of DEC in a cohort of 47 *W. bancrofti*-infected men in Tanzania. All study participants developed AEs, but the research group found no correlation between severity of AEs and IL-6 levels post-treatment (90). Increased post-treatment levels of IL-6 have also been associated with the development of AEs in onchocerciasis patients after treatment (84, 91). All the previously published studies that focused on AEs after LF-treatment had small sample sizes, and they used the standard of care for their time, instead of the current WHO recommended treatments for LF.

1.3.3 *Wolbachia as a cause of adverse events.*

*Wolbachia* has been hypothesized to be involved in the development of post-treatment AEs in LF-infected individuals since its re-discovery in these parasites in 1999. A popular hypothesis to explain AEs is that the *Wolbachia* endosymbiont are released from dying filarial worms and Mf after treatment, and that this is the main cause of AEs. A study that supports this scenario measured *Wolbachia* DNA in human plasma before and after treatment in *B. malayi* infected adults treated daily for 12 days with DEC. Post-treatment blood was collected at 2, 4, 8, 24, 32, 48 and 120 hours after the first dose, and it was concluded that people with moderate and severe AEs had more *Wolbachia* positive post-treatment samples when compared to people with mild or no AEs. This study also
concluded that the DNA was from whole bacterial cells and not free DNA, because after the plasma was centrifuged Wolbachia DNA was only detected in the pellet (92).

A more detailed model for how *Wolbachia* induces AEs was introduced by Taylor et al. in two papers from 2000-2001, where the authors state that *Wolbachia* can release LPS-like molecules that activate the innate inflammatory response through toll like receptors (TLRs) leading to either the symptoms of chronic infections or the AEs following treatment (Figure 1.6) (93, 94). This hypothesis was based on the discovery of a *B. malayi* derived LPS-like molecule that was heat-stable, reacted positively in the *Limulus* amoebocyte lysate (LAL) assay, and could be inhibited by polymyxin B. This soluble extract is a potent inducer of TNF-α, IL-1β and nitric oxide (NO) in murine macrophages. *B. malayi* extract cannot induce these cytokines in macrophages from LPS-nonresponsive C3H/HeJ mice (TLR4 defective due to mutation), suggesting that signaling occurs through TLR4. Additionally, extracts from the filarial worm *A. viteae* (no *Wolbachia*) do not elicit this pro-inflammatory response in macrophages, and did not react positively in the LAL assay, indicating that the LPS-like molecule in the *B. malayi* extracts originated from *Wolbachia*. Live filarial parasites and culture supernatants failed to elicit inflammatory responses from macrophages, so they hypothesized that the *Wolbachia* substrate is only released from dying worms. The Taylor group later revised their initial hypothesis and replaced TLR4 with TLR2-TLR6 in their proposed *Wolbachia* LPS-like signaling pathway (95). In this publication they used cell lines transfected with human TLRs and macrophages from TLR and adapter molecule deficient mice, and observed TLR2-TLR6 activation by the *Wolbachia* LPS-like molecule utilizing the mediators MyD88 and TIRAP/Mal. From the literature it is known that TLR2 can bind
bacterial lipoproteins, LPS and other bacterial components and activate NF-κB that in
turn induces pro-inflammatory cytokines such as IL-6 (96, 97).

Interestingly, the *B. malayi*-associated *Wolbachia* genome was published in 2004-
2005 (98, 99), and it did not include the homologs of the genes responsible for the
biosynthesis of lipid A (a component of LPS). It is therefore unlikely that *B. malayi
Wolbachia* contains LPS in its cell wall (99). However, the published *Wolbachia* genome
and bioinformatics methods have been used to predict the presence of two putative
candidate *Wolbachia* lipoproteins: peptidoglycan-associated lipoprotein (PAL), and a
type IV secretion system protein (VirB6) (100). In this same publication Turner et al.
conclude that a synthetic, lipolated version of the N-terminus of *Wolbachia* PAL, WoLP,
can signal through TLR2-TLR6, and induce pro-inflammatory responses *in vitro* (murine
and human cells) and *in vivo* (mice). In a 2014 publication, Tamarozzi et al. conclude that
WoLP can activate human neutrophils *in vitro* as shown by changes in their cell shape
and IL-8 production upon exposure to this stimulus (101). Recently a proteomics
approach has confirmed the presence of PAL in extracts of *B. malayi*, and it was one of
the most abundant proteins found in extracts from adult female worms. Additionally
immunogold labeling showed that the lipoprotein was localized in the bacterial
membrane (102). There is still insufficient evidence *in vivo* to show that endosymbiotic
bacteria provide a pro-inflammatory stimulus, and there is no information about levels of
this lipoprotein/LPS-like substance is post-treatment plasma of LF-infected individuals.

The major surface protein of *Wolbachia* (WSP) has also been identified to
activate the innate immune response through TLR signaling (103). In this study, cultured
whole blood cells from *O. volvulus* infected individuals were stimulated with purified
recombinant WSP, and found to release TNF-α, IL-12 and IL-8 in response to stimulation. These results were confirmed in PMBCs isolated from 3 healthy Europeans, where rWSP strongly stimulated the release of TNF-α, IL-1β, IL-6, and IL-8. This immune response was dependent on TLR2 and TLR4. WSP has been shown to be antigenic, and WSP-specific antibodies have been identified in human sera from infected individuals, with CP patients having the highest level of antibodies (104).

In a study from 2008 Supali et al. demonstrated that a six-week treatment course of doxycycline before DEC/ALB anti-filarial treatment decreased the risk of AEs (105). This was a double-blind, randomized, placebo-controlled field trial of 161 B. malayi infected persons. After the doxycycline treatment the Wolbachia load was decreased by 98%, and AEs following DEC/ALB were less in the group that received pre-treatment doxycycline when compared to those who received pre-treatment placebo. However, at the one-year follow-up microfilaremia was reduced by 87.5% in patients receiving both doxycycline and DEC/ALB, but only 26.7% in individuals receiving only DEC/ALB, so it is unclear if the decrease in AEs was due to decreased Mf loads or decreased Wolbachia after the doxycycline treatment.

An onchocerciasis mouse model has been used to study the role of Wolbachia in eliciting pro-inflammatory pathways causing corneal inflammatory pathology (106). In this study various filarial worm extracts were injected into the murine corneal stroma, and the resulting pathology scored. It was concluded that extracts from doxycycline treated worms (decreased Wolbachia) induced less pathology in the cornea when compared to non-treated worm extracts. The group also concluded that Wolbachia containing extracts from B. malayi resulted in more inflammation when compared to A. viteae extracts.
Finally, the LPS-hyposensitive C3H/HeJ mice demonstrated less pathology indicating signaling through TLR4. The previously mentioned WoLP has also been shown to induce corneal inflammation using this model (100).

It is difficult to attribute all AEs following anti-filarial treatment to Wolbachia. L. loa is a Wolbachia-free filarial nematode that infects humans, and it is responsible for the most severe post-treatment AEs observed after LF MDA. Individuals with high L. loa infection burdens can develop encephalopathy and die after IVM treatment, and this is not observed in LF patients (79). A group has reported that rodents infected with A. viteae develop severe AEs and die following effective microfilaricidal therapy, whereas animals infected with the Wolbachia-containing B. malayi tolerate corresponding treatment (107). They conclude that AEs might be caused by Mf-derived components different from Wolbachia-released factors.

1.3.4 Circulating immune complexes as a cause of adverse events.

Immune complexes (IC) are heterogeneous high molecular-weight aggregates of antigens, antibodies and components of the complement cascade (108), and when they circulate or accumulate in tissue they activate pro-inflammatory pathways. A paper from 1991 hypothesized that AEs following DEC or IVM treatment could be caused by immune complexes triggered by the release of filarial antigens following treatment (109). In this double-blind study 60 patients infected with W. bancrofti were randomized to receive a 14-day treatment of DEC or a single dose of IVM, and various immunological changes were followed after treatment. The results show that 24-48 hours after treatment antibodies to microfilarial excretory-secretory (ES) antigens decreased, with a concurrent
increase in filarial HC 11 antigens (HC 11 is a phosphorylcholine determinant present in all filarial worm stages), and these changes were temporally associated with the onset of AEs. The authors reported that the serological changes occurred in the majority of patients, but the magnitude was significantly greater in individuals who developed AEs. It is possible that the results might have been more significant and different earlier, if the data had been analyzed using only individuals with AEs.

A study from 1988 also concluded that filarial ES antigen titer increased after DEC treatment, and this group additionally observed an increase in IC titer after treatment (110). In this study 27 W. bancrofti-infected patients were treated for 12 days with DEC, and blood was collected before and 7 days after the first DEC dose. An anti C3 ELISA was used to measure ICs. The mean ES antigen titers increased from 732 to 1633 on day 7, whereas filarial-specific IgG and IgM antibody levels decreased during this time. Only 3 individuals had detectable filarial IC before treatment, but a sudden increase of mean IC titer of 73,020 at day 7 was observed in these individuals.

More recently Senbagavalli et al. hypothesized that the interaction of circulating immune complexes (CIC) and complement with the host innate immune system is a major contributing factor in the development of lymphatic pathological change and/or host resistance (111). In this paper from 2011 the authors determined the levels of CIC in the different clinical categories of LF (INF, CP and EN) in a total of 120 individuals. CICs were assayed by using a polyethylene glycol (PEG) precipitation method and an enzyme immunoassay (EIA), and irrespective of the method used INF had significantly ($P < 0.001$) higher levels of CIC than either CP or EN. This result contradicts previously published data that reports highest levels of CIC in CP individuals (112). This paper also
describes how cultured granulocytes from healthy controls respond by cytokine release when stimulated with PEG-precipitated IC from INF, CP and EN plasma. IC from INF and CP induced significantly higher levels of IL-6 and IL-17 when compared to EN. In contrast to the pro-inflammatory cytokines, IL-4, was reduced when granulocytes were incubated with IC from INF and CP compared to EN. No significant difference was found for other cytokines, such as TNF-α, IL-1β and IL-10.

1.3.5 **Host gene expression before and after treatment in lymphatic filariasis patients.**

There are no published human RNA sequencing data for LF infections. Semnani et al. used microarrays to examine the effect of treatment on gene expression in monocytes from LF patients (113). PBMCs were isolated from 4 *W. bancrofti*-infected individuals before treatment and at 8 months after IVM/ALB treatment. The results showed that 47 genes were repressed and 41 genes were induced in paired samples (pre-versus post-treatment). Utilizing hierarchical clustering they identified distinct sets of genes that were highly expressed pre-treatment and repressed post-treatment, and opposite. Genes induced post-treatment included many members of the heat shock protein (HSP) family with ATP binding activity, and genes involved in signal transduction, such as IL-1 receptor type I. Among the genes repressed in the post-treatment samples were those involved in signaling (chemokine receptor 7, mitogen-activated protein kinase 8, Epstein-Barr virus-induced gene 2), transcription and protein metabolism. Interestingly, this study also examined the difference in gene expression between monocytes from LF infected and un-infected individuals. Only 62 genes (8 repressed and 54 induced in *W. bancrofti*-infected individuals) out of the possible 30,000
were significantly different in monocytes of \textit{W. bancrofti}-infected versus \textit{W. bancrofti}-uninfected individuals. The genes over-expressed in LF patients were involved in apoptosis (IL-1β, BCL2A1, and MOAP1) and cell adhesion. Treatment with single-dose IVM plus ALB normalized monocyte gene expression at 8 months. Host gene expression has not been correlated to the development of AEs after treatment for LF.

1.4 Obstacles and resources for studying adverse events after anti-filarial treatment.

1.4.1 Obstacles.

\textit{In vitro} culture systems and convenient animal models are not available for the most clinically relevant and important filarial nematodes, \textit{W. bancrofti} and \textit{O. volvulus}. \textit{B. malayi} and the \textit{Wolbachia}-free \textit{A. viteae}, on the other hand, can be maintained in the laboratory in gerbils (114-116). The filarial nematode \textit{Litomosoides sigmondontis} normally infects cotton rats, but it can also be maintained in a mouse model (116). Even with these possible model systems, studying AEs in an animal model would be very complex. The drugs that cause AEs (IVM and DEC) only exert their anti-filarial effects \textit{in vivo} when they can work with the host immune system. It is unclear how mouse or gerbil immune systems differ in regards to treatment efficacy and AE rates and presentation. Another problem would be how to measure AEs in the animal. Therefore, in my thesis I focus only on the study of AE pathogenesis in humans, and this approach of course has its own limitations. It is impossible to control for all possible variables in a human sample set. For example, gene expression can be influenced by age, sex, diet, season, general health status and a wealth of other factors. In order to minimize these confounding variables as much as possible, we decided to look at the change in gene
expression (and filarial components) in the same individuals just before treatment and at 24 hours post-treatment. This short 24-hour timeframe minimizes the effects of other variables; therefore treatment and/or the development of AEs is the main factor that influences the change in gene expression during this time. We also collected as much information as possible for each individual including detailed clinical data, so a metadata analysis could be completed.

1.4.2 Resources.

The most crucial stage of this project was to collect appropriate human samples in order to test our hypotheses. Our group is spearheading the broad research project Death to Onchocerciasis and Lymphatic Filariasis (DOLF, www.dolf.wustl.edu). DOLF is currently involved in clinical and community trials in over ten LF-endemic countries, and this has allowed me to access valuable human samples and clinical data. Additionally, our group has developed a long-lasting collaboration with Dr. Mitreva’s group at the McDonnell Genome Institute (MGI) at Washington University, which has been invaluable during the analysis stage for the complex RNA-seq data.

1.5 Aims and scope of thesis.

It is well established that AEs occur commonly in LF-infected individuals after treatment, but the pathogenesis is still not completely understood. State of the art comprehensive studies that considered both filarial and host involvement in AE development were lacking. In this study we therefore examined the possible immune-stimulatory filarial components that are released post-treatment, and the host immune
response during AEs. The objective of this thesis was to characterize the host response during post-treatment AEs, and to identify possible parasite components that trigger AEs. We hypothesized that AEs are associated with unique host response patterns, and that filarial and/or Wolbachia components are released post-treatment and these foreign antigens and/or CIC can trigger AEs.

The fear of AEs after treatment is an important factor leads to non-compliance with LF MDA programs. Understanding the underlying mechanisms for the development of AEs can help to improve management, and increase the compliance to MDA programs, and this can have a positive impact on millions of LF infected people that will be treated over the next many years. This will be particularly important for the new triple therapy, because this treatment might become more mainstream within the next years. We will therefore be on the forefront with innovative and relevant data on the pathogenesis of AEs related to this treatment. This data will be very important because in the preliminary studies over 50% of LF of infected individuals that received the triple therapy developed AEs (57).
1.6 References.


22. LeAnne M Fox CLK. *Hunter's Tropical Medicine and Emerging Infectious Disease.* Elsevier; 2013.


Figure 1.1: Historical background of lymphatic filariasis.

Figure 1.2: Life cycle of *Wuchereria bancrofti*.

This figure is available on the Centers for Disease Control and Prevention website (https://www.cdc.gov/parasites/lymphaticfilariasis/biology_w_bancrofti.html).
Figure 1.3: Distribution of *Wolbachia* endosymbionts in adult filarial worms.

Longitudinal cross-section of mature [A] and immature [B] adult female filarial worm with *Wolbachia* highlighted as red dots. [C] Longitudinal cross-section of mature adult male filarial worm with *Wolbachia* highlighted as red dots.

Figure 1.4: Images of clinical manifestations of lymphatic filariasis.

Figure 1.5: The progress of GPELF (Global Program to Eliminate Lymphatic Filariasis).

[A] The cumulative number of countries that have started an LF mass drug administration control program from the year 2000 to 2014. [B] The cumulative number of anti-LF treatments delivered by GPELF from the year 2000 to 2014. Data is from the World Health Organization’s (WHO) PCT databank (Lymphatic filariasis), and the values in black represent the data that was not included in the previous published analyses (55, 117). Insert in [B] represents the proportion of the treatments delivered to each of the WHO regions (AMRO: Region of the Americas, AFRO: African Region, EMRO: Eastern Mediterranean Region, WPRO: Western Pacific Region, and SEARO: South-East Asia Region).

Figure 1.6: *Wolbachia* signaling pathway.

An overview of the proposed mechanisms by which *Wolbachia* contributes to the pathogenesis of lymphatic filarial disease and adverse events after treatment. Figure is adapted from Taylor MJ, Cross HF, Ford L, Makunde WH, Prasad GB, and Bilo K. Wolbachia bacteria in filarial immunity and disease. *Parasite Immunol.* 2001;23(7):401-9.
Chapter 2:

Changes in cytokine, filarial antigen, and DNA levels associated with adverse events following treatment of lymphatic filariasis
Preface

BJA designed and performed the experiments, analyzed data, created figures, and wrote the manuscript. GJW and PUF supervised the work, assisted in experiment design and data analysis and assisted with manuscript preparation. JK and KC assisted with experiments. NS and SS collected samples in the field. CLK supervised the clinical trial.

This chapter is adapted from a manuscript published in the Journal of Infectious Diseases:

2.1 Summary.

Background: Mild to moderate adverse events (AEs) are common after treatment of lymphatic filariasis (LF) and pose a major challenge for the global LF elimination program. We studied changes in cytokine levels and filarial worm components in plasma of subjects with and without AEs following treatment of LF.

Methods: Participants (N=24) were hospitalized and monitored for AEs following treatment. Cytokines (27), filarial DNA, circulating filarial antigen (CFA), and immune complexes were measured in plasma samples collected before and after treatment.

Results: Levels for 16 cytokines increased after treatment in individuals with moderate AEs compared to individuals with no and/or mild AEs. These included three major pro-inflammatory cytokines (IL-6, TNF-α and IL-1β). Eotaxin-1 levels were elevated at baseline in individuals who developed moderate AEs after treatment; thus eotaxin-1 is a potential biomarker for AE risk. CFA and filarial DNA levels increased more in individuals with moderate AEs after treatment than in people with no/mild AEs.

Conclusions: Increases in cytokine, filarial DNA and CFA levels were associated with development of AEs following treatment of LF. Improved understanding of the pathogenesis of AEs may lead to improved methods for their prevention or management that could increase compliance in elimination programs.
2.2 Introduction.

Lymphatic filariasis (LF) is a neglected tropical disease that is caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. Adult worms release first stage larvae (microfilariae or Mf) into the blood, and these are ingested by mosquitoes. The parasites develop to become infective third stage larvae that can initiate new infections when they are transmitted to humans by mosquitoes. The worms and the host’s inflammatory responses can lead to severe morbidity with lymphedema, hydrocele, and elephantiasis (1).

The World Health Organization (WHO) launched the Global Program to Eliminate LF (GPELF) in the year 2000 with the goal of eliminating LF as a public health problem by 2020. The primary tool used by GPELF is annual mass drug administration (MDA), and some 500 million people are treated each year (2). The medications used, namely albendazole (ALB), ivermectin (IVM) and diethylcarbamazine (DEC), have well-established safety profiles, and serious adverse events (AEs) related to treatment are very rare. However, mild to moderate AEs such as fever, and headache are common. High MDA compliance is important for LF elimination programs (3). The fear of AEs in communities receiving MDA reduces compliance (4, 5). Understanding the pathogenesis AEs is even more urgent at this time, because recent studies have shown that a single dose of all three LF MDA drugs (IVM, DEC and ALB, sometimes called “IDA”) is more effective for clearing Mf than the current two-drug MDA regimens ((6) and authors’ unpublished observations). This increased efficacy may be associated with increased AE rates in infected individuals.

The pathogenesis of AEs after treatment of LF is poorly understood. Host immune responses and parasite death are believed to be involved, because post-treatment AE rates are much higher in infected individuals, and because AE rates are correlated with blood Mf
counts (7). AEs are probably related to release of parasite antigens and/or *Wolbachia* (an intracellular alpha proteobacteria found in LF-causing filarial worms). *Wolbachia*-derived molecules may interact directly with the innate immune system through ligands such as Toll-like receptors (TLRs) to activate immune cells to release cytokines (8-13). Few studies have looked at changes in cytokines associated with AEs following LF treatment. One study documented increases in IL-6 and TNF-α after treatment in five men with AEs (14). Another study reported that AEs were associated with increased levels of IL-6, lipopolysaccharide binding protein (LBP), IL-10 and soluble TNF receptor (15). Other studies suggested that AEs may be related to circulating immune complexes (CIC) that form when filarial antigens are released by dying parasites (16, 17). CIC from plasma of LF-infected individuals have been shown to be pro-inflammatory when added to granulocytes (18). A recent clinical trial provided us with the opportunity to use 21st century methods to revisit the issue of AE pathogenesis.

2.3 Methods.

Study design.

Plasma samples used for this study were obtained during a pharmacokinetic trial conducted in Papua New Guinea (PNG) in 2013 (6). Twenty-four *W. bancrofti* infected individuals were randomized to one of two treatment arms: the standard LF MDA regimen for PNG (ALB and DEC) or the new IDA triple therapy regimen. The AE assessment protocol was described in a previous publication (6). Briefly, objective AEs were assessed (vital signs and a brief physical exam) for all study participants at 0, 4, 8, 12, 24, 48 and 72 hours post-treatment in a hospital setting. Subjective AEs were
assessed at the same times by asking the participants open-ended questions about symptoms that developed after treatment. All participants were followed as outpatients and examined on day 7. Nineteen of 24 participants (79%) developed at least one AE, and 7 of these individuals had fevers greater than 38°C. Blood was collected immediately before treatment and at 11 time-points after treatment (1hr to 72hrs). Notable in this study was the high Mf levels (geometric mean = 1,679, range 133-13,776 Mf/mL). Plasma samples were stored and shipped at -80°C. Informed consent was obtained from all participants as previously described (6).

**Adverse events classification.**

AEs were scored as none, mild, or moderate. Those with moderate AEs (N=7) had at least one new symptom plus objectively measured fever (a temperature of ≥ 38°C) within 72 hours after treatment. Individuals with subjective or objective AEs without fever were considered to have mild AEs (N=12). Individuals with no objective or subjective symptoms were considered to have no AEs (N=5).

**Cytokine assay.**

Twenty-seven cytokines were measured using a MAGPIX system with the Bio-Plex Human 27-Plex Cytokine Panel and Bio-Plex Cytokine Reagent Kit (Bio-Rad, Hercules, CA). Plasma samples were thawed and centrifuged before testing. A preliminary study tested samples from all 12 time-points (pre-treatment and 1, 2, 3, 4, 6, 8, 12, 24, 36, 48 and 72 hours post-treatment) for 7 participants. Since there were no changes in cytokine levels during the first 6 hours, only 7 time points (pre-treatment, 8,
12, 24, 36, 48 and 72 hours post-treatment) were tested for the remaining 17 study participants. All samples were tested in duplicate, and all samples from the same individual were run on the same plate. The cytokine assay panel included IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin-1, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF. Standard curves were calculated using the manufacturer’s software, and our analysis considered mean concentrations (pg/mL) from two duplicate wells. Mean levels for all 27 cytokines were calculated for each AE group at each time-point. Kruskal-Wallis H tests were used to compare absolute cytokine levels between the three AE groups at each time-point. Wilcoxon signed-rank tests were used to compare post-treatment levels to baseline levels within AE groups for each time-point. After evaluation of the data three outliers with extremely elevated levels at baseline were excluded from the analysis.

**Circulating Filarial Antigen (CFA).**

A direct sandwich enzyme immunoassay (EIA) was performed as previously described (19, 20). Plasma samples were available for 21 of the 24 individuals for this test (6 moderate, 10 mild, and 5 no AEs), and samples from 5 time-points were tested (pre-treatment, 6, 12, 24 and 48 hours post-treatment). All samples from individual participants were tested in duplicate on the same plate. The mean CFA levels were calculated for each AE group at each time-point. Kruskal-Wallis H tests were used to compare the absolute CFA levels between the three AE groups at each time-point.
Wilcoxon signed-rank tests were used to compare post-treatment CFA levels at each time-point to baseline levels within the AE groups.

**Detection of filarial DNA by qPCR.**

DNA was extracted from 100μL of plasma using the E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Norcross, GA) using the manufacturer’s protocol. The qPCR assay was a TaqMan probe-based assay, and the target was the “long DNA repeat” of *W. bancrofti* (LDR; GenBank accession no. AY297458). We used previously published primers and probes (21) purchased from Integrated DNA Technologies (Coralville, IA). Real-time PCR reactions were performed with 10μL of TaqMan master mix (Applied Biosystems, Foster City, CA) plus 450 nmol/L of primers, 125 nmol/L probe, and 2μL DNA with a final volume of 20μL. Thermal cycling was performed with a QuantStudio 7-Plex Real-Time PCR System (Applied Biosystems). PCR reactions were carried out for 40 cycles, and cycle threshold (Ct) values were determined using the manufacturer’s software. Plasma samples were available for 21 of the 24 individuals for this assay (7 moderate, 9 mild, and 5 no AEs), and 7 time-points were selected (pre-treatment, 8, 12, 24, 36, 48 and 72 hours post-treatment). Samples were run in duplicate, and all samples from individual participants were tested on the same plate. Each plate contained a positive control (DNA extracted from *W. bancrofti* Mf), and two negative controls (DNA extracted from plasma samples from healthy North American control subjects and deionized water). Delta Ct values (baseline Ct value minus post-treatment Ct value) were calculated at each time-point for each individual, and one-way ANOVA analysis was used to compare the delta Ct values between the three AE groups at each time-point.
Immune complex assay.

In this assay CIC were incubated with human C1q (part of the first component in the classical complement pathway) that was immobilized on microtiter plates. C1q was purchased from Sigma-Aldrich, St. Louis, MO. Nunc Immulon 2HB flat-bottom 96-well plates (Thermo Scientific, Waltham, MA) were coated with 50µL of 0.01mg/mL C1q in 1x PBS pH7.4 and incubated at 4°C overnight. Plates were washed and blocked for 1 hour at room temperature (RT). After washing, 50µL sample plasma or standard (diluted 1:60 in PBS with 0.5% casein, 0.5% Tween-20) was added to each well, and the plates were incubated at RT for 1 hour. Aggregated human gamma globulin (AHG) was used as the positive control and standard. Alkaline phosphatase-conjugated goat anti-human IgG was used at a dilution of 1:1000, and plates were incubated for 1 hour at 37°C. The plates were developed with alkaline phosphatase substrate (pNPP disodium salt hexahydrate) and read at 405nm. Plasma was available from 20 of the 24 individuals for this assay (7 moderate, 10 mild, and 5 no AEs), and 7 time-points were selected (pre-treatment, 8, 12, 24, 36, 48 and 72 hours post-treatment). Samples were run in duplicate and all samples from individual participants were run on the same plate. Each plate contained two negative controls (plasma samples from healthy North American control subjects and deionized water). Values were expressed as ng/mL of AHG, and mean CIC values were calculated for each AE group at each time-point. Kruskal-Wallis H tests were used to compare absolute CIC levels between the three AE groups at each time-point. Wilcoxon signed-rank tests were used to compare post-treatment CIC levels at each time-point to baseline levels within each AE group.
2.4 Results.

Cytokine levels.

Three of the 24 individuals (all in the mild AE group) had extremely elevated cytokine levels at baseline, and they were excluded from the analysis. These three individuals had outlier levels for 9, 12 and 14 of the 27 measured cytokines respectively, at baseline. These high baseline cytokine levels were unrelated to treatment and their inclusion would have distorted the data, because the aim of the study was to investigate the change in cytokines post-treatment.

Changes in cytokine levels after treatment were significantly different in persons with moderate AEs compared to those in persons with no and/or mild AEs for 22 of the 27 cytokines tested (Table 2.1) (Kruskal-Wallis analysis followed by post-hoc tests to determine which AE groups were significantly different). Most of these cytokines (IL-1β, IL-1Ra, IL-4, IL-6, IL-7, IL-10, IL-12, IL-17, G-CSF, IP-10, MCP-1, MIP-1α, PDGF-BB, MIP-1β, TNF-α, and VEGF) increased significantly more in the moderate AE group after treatment compared to the no and/or mild AE groups. IL-6, IL-10, MCP-1 and MIP-1β had the most dramatic post-treatment increases in the moderate AE group (Figure 2.1). Several cytokines (IL-8, IL-13 and eotaxin-1) were significantly higher at baseline in persons who developed moderate AEs. Eotaxin-1 was greatly increased pre-treatment in 6 out of the 7 individuals who later developed moderate AEs (Figure 2.2). Baseline eotaxin-1 levels were not correlated with baseline Mf counts. The finding that some cytokines were higher at baseline in individuals who would go on to develop AEs was surprising, and we decided to redo the analysis including the three excluded individuals. There was still a significant difference in baseline eotaxin-1 and IL-8 levels.
between individuals with moderate AEs compared to individuals with no and/or mild AEs. However the difference in IL-13 at baseline disappeared when the outliers were included.

Values for several cytokines were lower at baseline (RANTES) or decreased significantly post-treatment (IL-2 and IL-15) in people who developed moderate AEs compared to people with no and/or mild AEs. Five cytokines (IL-5, IL-9, FGF-basic, GM-CSF and IFN-γ) did not differ by AE group at any time-point, however IL-5 did increase post-treatment as previously described (22, 23). We did not correct for multiple comparisons, so additional data would be needed to confirm our findings. However, the differences in cytokine levels were dramatic between the AE groups, and they persisted over time making the results more credible. Based on the standard significance level of 0.05, approximately 3 differences would be expected to be significant by chance for 54 tests (27 cytokines measured at 2 time-points). We found 28 significant differences in cytokines levels at 12 and 24 hours post-treatment.

**CFA levels and Mf counts.**

CFA is known to circulate in the blood of LF-infected individuals, and this antigen is used as a diagnostic marker. The detection limit of the CFA EIA assay is 6.3ng/mL. CFA was detected in all samples from all study subjects. Baseline CFA levels were positively correlated with baseline Mf counts (Spearman’s rho: 0.66, P = 0.001), and absolute CFA levels were significantly higher at baseline in the individuals who later developed moderate AEs. CFA levels were significantly higher in the moderate AE group compared to the no/mild AE groups at all time-points (Figure 2.3). CFA levels increased
in all groups after treatment, but the difference was only significant at the 48hr time-point 
\( (P = 0.048 \text{ by Wilcoxon signed-rank test} ) \) compared to the baseline level in the moderate 
AE group. There was no difference in absolute CFA levels between the two treatment 
arms at any time-point.

Baseline Mf counts were higher in individuals who developed moderate AEs 
(geometric mean 4491 Mf/mL compared to 1111 Mf/mL in the mild AE group and 1351 
Mf/mL in the no AEs group), and this difference was significant \( (P = 0.017 \text{ by the } \) 
Kruskal-Wallis test).

**Filarial DNA levels.**

Thirty-three percent of participants had detectable filarial DNA in plasma 
collected before treatment. There was no correlation between pre-treatment Ct values and 
baseline Mf counts (Spearman’s rho: -0.1). Filarial DNA was detected in plasma of all 
subjects at 8 hours post-treatment. Also, filarial DNA levels increased (Ct values 
decreased) after treatment in persons with filarial DNA detected at baseline. DNA levels 
quickly increased for the first 12-24 hours after treatment, after which they start to 
decrease. However, filarial DNA was still detectable in plasma in 95% of individuals 
72hr post-treatment. There was a statistically significant difference in delta Ct values 
between the three AE groups at 12 and 24 hours post-treatment, and this difference was 
due to significantly higher delta Ct values (larger increase in DNA levels) in the moderate 
AE group compared to the mild AE group (Figure 2.4). A similar trend was observed 
between the moderate and no AE groups, but the difference was not statistically 
significant due to the small number of individuals in the no AE group (N=5). There were
no significant differences in filarial DNA levels in plasma by treatment arm at any time-point.

**CIC levels.**

CIC were detected in all samples, and the range at baseline was 108-1312 ng AHG equivalent/mL (median 417 ng). Baseline CIC levels were positively correlated with Mf counts (Spearman’s rho: 0.68, $P = 0.006$). There was no difference in absolute CIC levels between the AE groups at any time-point (Supplemental Table 2.1). CIC levels were relatively stable after treatment, and there were no consistent patterns as differences included both increases and decreases.

### 2.5 Discussion.

The aim of this study was to determine the role of host cytokines, and filarial components released by parasites after treatment, in the development of AEs in LF-infected individuals. This is the most detailed study to date of cytokine responses that occur in persons with AEs after treatment of LF. We identified 22 cytokines that were differentially regulated post-treatment between the three AE groups. The majority of these cytokines increased more post-treatment in persons who developed moderate AEs. Our data are consistent with previously published results such as increases in IL-6, TNF-$\alpha$ and IL-10 post-treatment in people with AEs (14, 15). However, we also found that many other cytokines increased in participants with moderate AEs.

Plasma levels of filarial DNA and CFA both increased after treatment, and post-treatment increases in CFA were much more dramatic in people who developed moderate
AEs than in those with no/mild AEs. Treatment with ALB/DEC and IVM/DEC/ALB resulted in similar changes in CFA and filarial DNA levels after treatment, suggesting that the two treatments have similar activity against Mf and adult filarial worms in the first days after treatment. CFA levels increased slightly later after treatment than filarial DNA, but the CFA increases persisted for a longer time. This result is consistent with prior reports of increased CFA levels 5-7 days post-treatment (24, 25). Taken together, our results suggest that cytokines, filarial DNA, and CFA all may be involved in the pathogenesis of AEs. It is likely that the cytokine responses are triggered by molecules that are released from dying parasites. However, it is unclear whether cytokine release is triggered by phagocytosis of parasite debris or by direct interaction of parasite molecules with ligands on the surface of host cells. Compared to filarial DNA and CFA levels, CIC levels were stable after treatment, and there was no difference in CIC levels between the AE groups at any time-point. These results suggest that CIC may not be involved in the pathogenesis of moderate AEs.

Cytokine changes have been extensively studied in patients with septicemia or after exposure to endotoxin with sequential increases in TNF-α, IL-1β, IL-6, IL-1Ra and IL-10 (26). The cytokine pattern in this study was somewhat similar in that TNF-α was the first to increase with a peak at 8hr post-treatment. IL-6 had the largest increase (a 13-fold increase in the moderate AE group) with a peak at 12hr. IL-10, which increases later after endotoxin exposure was not delayed in this study; it rose by 8hr and peaked at 12hr. Increases in pro-inflammatory cytokines may be stimulated by release of Wolbachia from dying filarial worms, but additional studies will have to be conducted to test this hypothesis. Dramatic increases in MCP-1 (monocyte chemoattractant protein 1) and
MIP-1β/1α (macrophage inflammatory protein 1β/1α) in persons with moderate AEs suggest that monocytes and/or macrophages are involved in the pathogenesis of AEs. Both of these cytokines are released by macrophages after endotoxin exposure (27), so this finding is consistent with the *Wolbachia* release hypothesis. Although *Wolbachia* do not contain lipopolysaccharide, they do contain endotoxin-like lipoproteins that interact with cellular ligands TLR2 and TLR6 (9, 11, 13).

Several baseline characteristics are known to be correlated with development of AEs after treatment of LF. For example, high Mf counts are a known risk factor (7), and our study confirmed that this is the case. High CFA levels have not been previously identified as a risk factor for AEs. Perhaps because antigen levels were not measured. However, the association is not surprising, because CFA levels are positively correlated with Mf counts. The finding that participants with elevated levels of certain cytokines prior to treatment were at increased risk for moderate AEs was not anticipated. This was especially true for eotaxin-1, a chemokine secreted by various cells that attracts circulating eosinophils to their respective tissue (28). One potential explanation for this finding is that individuals with high levels of eotaxin-1 may have activated eosinophils that are poised for attack. These cells rapidly kill parasites that have been damaged by anthelmintic drugs and this may trigger a more vigorous pro-inflammatory response that results in AEs. This hypothesis is supported by the finding that eotaxin-1 deficient mice have reduced eosinophil responses to TLR2 activation and filarial antigen exposure, and the finding that macrophages from these mice produce less IL-6 (29). Additional research will be needed to understand the apparent link between eotaxin-1 and AEs.
Samples from the PNG trial were ideal for our study because of the high rate of AEs, the detailed clinical information available for participants, and the availability of plasma at many time-points after treatment. One limitation is that we were unable to assess Wolbachia in the plasma samples for technical reasons. Another limitation is that the correlations we observed do not prove causation.

In conclusion, this study has provided additional information on changes in plasma cytokine levels and in filarial worm components that are associated with moderate AEs after LF treatment. We have also shown that high Mf counts and high levels of CFA and eotaxin-1 at baseline are associated with increased risk for moderate AEs. Taken together, our results suggest that components released from filarial worms interact with the host immune system to release pro-inflammatory cytokines that lead to moderate AEs with fever and associated symptoms. More work is needed to identify the specific filarial worm components that are responsible for this immune activation, but Wolbachia endobacteria represent an attractive candidate. However, they are unlikely to be the only contributor, because similar AEs occur in patients after treatment of loiasis, and Loa loa does not contain Wolbachia (30). Improved understanding of the pathogenesis of AEs may lead to strategies to prevent or manage AEs in ways that increase compliance with MDA, which is essential for the success of LF elimination programs.

2.6 Acknowledgements.

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New Guinea, for their support during sample collection. All authors report no conflict of interest.
2.7 References.


22. Gopinath R, Hanna LE, Kumaraswami V, Perumal V, Kavitha V, Vijayasekaran V, and
Nutman TB. Perturbations in eosinophil homeostasis following treatment of lymphatic

23. Limaye AP, Ottesen EA, Kumaraswami V, Abrams JS, Regunathan J, Vijayasekaran V,
Jayaraman K, and Nutman TB. Kinetics of serum and cellular interleukin-5 in
posttreatment eosinophilia of patients with lymphatic filariasis. J Infect Dis.
1993;167(6):1396-400.

HN, Sheriff MH, Perera CS, and Dissanaike AS. Prolonged clearance of microfilaraemia
in patients with bancroftian filariasis after multiple high doses of ivermectin or

25. Weil GJ, Lammie PJ, Richards FO, Jr., and Eberhard ML. Changes in circulating parasite
antigen levels after treatment of bancroftian filariasis with diethylcarbamazine and

1993;54(1-78.


2016;4(5).

and Hoerauf A. Eotaxin-1 is involved in parasite clearance during chronic filarial

Serious reactions after mass treatment of onchocerciasis with ivermectin in an area
Figure 2.1: Cytokine levels pre- and post-treatment in individuals with no, mild and moderate adverse events (AEs).

Mean cytokine levels (± SE) in the three AE groups over time. IL-6 [A], IL-10 [B], MCP-1 [C] and MIP-1β [D] increased post-treatment in the moderate AE group while there were no significant changes in the no or mild AE groups. Significance (Kruskal-Wallis H Test): * represents $P < 0.05$, ** represents $P < 0.01$. 
**Figure 2.2:** Eotaxin-1 levels pre- and post-treatment in individuals with no, mild and moderate adverse events (AEs).

Mean eotaxin-1 levels (± SE) for all three adverse event (AE) groups over time. Eotaxin-1 levels were significantly higher at all time-points in the moderate AE group compared to the no and/or mild AE groups. Significance (Kruskal-Wallis H Test): * represents $P < 0.05$, ** represents $P < 0.01$. 
Figure 2.3: Circulating filarial antigen (CFA) levels pre- and post-treatment in individuals with no, mild and moderate adverse events (AEs).

Mean Wb (*W. bancrofti*) CFA levels (± SE) for each AE group over time. CFA levels were significantly higher in the moderate AE group than in the no and mild AE groups. Significance (Kruskal-Wallis H Test): * represents \( P < 0.05 \), ++ represents \( P < 0.01 \). CFA levels were significantly higher at 48 hours post-treatment compared to baseline in the moderate AE group (P-value= 0.048 by Wilcoxon signed-rank test).
Figure 2.4: Filarial DNA levels pre- and post-treatment in individuals with no, mild and moderate adverse events (AEs).

Filarial DNA levels in plasma (expressed as delta Ct ± SE) after treatment by adverse event (AE) group. Ct values decreased after treatment in all three AE groups, signifying an increase in filarial DNA levels in plasma. Mean delta Ct values were significantly greater in the moderate AE group when compared to the mild AE group at 12 ($P = 0.025$) and 24 ($P = 0.020$) hours post-treatment (by ANOVA).
Table 2.1: Changes in cytokines at different times after treatment in persons who developed moderate adverse events (AEs) after treatment of filariasis compared to those with no or mild AEs.

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<th>IL-1RA</th>
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<th>IL-4</th>
<th>IL-6</th>
<th>IL-7</th>
<th>IL-8</th>
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<th>MCP-1</th>
<th>MIP-1α</th>
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<th>TNF-α</th>
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*Hours post-treatment.

These differences were due to significant increases (represented with plus signs) in cytokine levels in the moderate AE group compared to the no and/or mild AE groups (Kruskal-Wallis analysis followed by post-hoc tests to determine which AE groups were statistically different). IL-2, IL-15 and RANTES were exceptions as these cytokines were decreased (represented with minus signs) before and/or after treatment in the moderate AE group. Significance (by the Kruskal-Wallis H Test): + or - corresponds to \( P < 0.05 \); ++ or - - corresponds to \( P < 0.01 \).
**Supplemental Table 2.1:** Levels of circulating immune complexes (CIC) in plasma at different times after treatment in the three adverse events (AEs) groups.

<table>
<thead>
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<th>Hours Post-Treatment</th>
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<th>24</th>
<th>36</th>
<th>48</th>
<th>72</th>
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<td>512</td>
<td>539</td>
<td>510</td>
<td>514 (95)</td>
<td>587</td>
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<td>(N=7)</td>
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<td>(146)</td>
<td>(158)</td>
<td>(207)</td>
<td>(162)</td>
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<tr>
<td><strong>Mild AEs</strong></td>
<td>373</td>
<td>384 (96)</td>
<td>378 (90)</td>
<td>391 (90)</td>
<td>386</td>
<td>361</td>
<td>396 (110)</td>
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<td>(N=10)</td>
<td>(96)</td>
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<td></td>
<td></td>
<td>(102)</td>
<td></td>
<td>(207)</td>
</tr>
<tr>
<td><strong>No AEs</strong></td>
<td>611</td>
<td>615</td>
<td>635</td>
<td>699</td>
<td>702</td>
<td>698</td>
<td>648 (151)</td>
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<td>(N=5)</td>
<td>(175)</td>
<td>(129)</td>
<td>(154)</td>
<td>(139)</td>
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Mean CIC plasma concentration, ng aggregated human immunoglobulin equivalent/mL (± SE).
Chapter 3

Systems analysis-based assessment of post-treatment adverse events in lymphatic filariasis
Preface

BJA designed research studies, conducted experiments, acquired data, analyzed data, and wrote the manuscript. BAR acquired and analyzed data. JK, MIH and KC conducted experiments. AM designed research studies (clinical trial). TS acquired field data. CLK designed research studies (clinical trial). MM designed research studies (RNA-seq). GJW and PUF designed research studies and wrote the manuscript.

This chapter is adapted from a manuscript published in PLOS Neglected Tropical Diseases:

3.1 Summary.

Background: Lymphatic filariasis (LF) is a neglected tropical disease, and the Global Program to Eliminate LF delivers mass drug administration (MDA) to 500 million people every year. Adverse events (AEs) are common after LF treatment.

Methodology/Principal Findings: To better understand the pathogenesis of AEs, we studied LF-patients from a treatment trial. Plasma levels of many filarial antigens increased post-treatment in individuals with AEs, and this is consistent with parasite death. Circulating immune complexes were not elevated in these participants, and the classical complement cascade was not activated. Multiple cytokines increased after treatment in persons with AEs. A transcriptomic analysis was performed for nine individuals with moderate systemic AEs and nine matched controls. Differential gene expression analysis identified a significant transcriptional signature associated with post-treatment AEs; 744 genes were upregulated. The transcriptional signature was enriched for TLR and NF-κB signaling. Increased expression of seven out of the top eight genes upregulated in persons with AEs were validated by qRT-PCR, including TLR2.

Conclusions/Significance: This is the first global study of changes in gene expression associated with AEs after treatment of lymphatic filariasis. Changes in cytokines were consistent with prior studies and with the RNAseq data. These results suggest that Wolbachia lipoprotein is involved in AE development, because it activates TLR2-TLR6 and downstream NF-κB. Additionally, LPS Binding Protein (LBP, which shuttles lipoproteins to TLR2) increased post-treatment in individuals with AEs. Improved understanding of the pathogenesis of AEs may lead to improved management, increased MDA compliance, and accelerated LF elimination.
3.2 Introduction.

Lymphatic filariasis (LF) is a disabling neglected tropical disease that is caused by the mosquito-borne filarial parasites *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. Adult worms live in the human host’s lymphatic system and release larval parasites (microfilariae or Mf) that circulate in the blood. Infection and host inflammatory responses to the parasite can lead to severe morbidity including lymphedema, hydrocele and elephantiasis (1). To combat this disease the WHO launched the Global Program to Eliminate Lymphatic Filariasis (GPELF) in the year 2000 with the goal of eliminating LF as a public health problem by 2020. The program uses mass drug administration (MDA), to cure infections, prevent disease, and reduce transmission of new infections. As of 2016 a total of 6.7 billion treatments had been delivered to more than 850 million individuals (2), making GPELF the largest public health intervention for an infectious disease to date based on MDA. Drugs used for LF MDA include albendazole (ALB), ivermectin (IVM) and diethylcarbamazine (DEC). MDA with two-drug combinations is usually provided annually for 4-6 years. The combinations used are ALB with IVM in sub-Saharan Africa and ALB with DEC in other regions (1). New studies have shown that combining all three drugs increases the anti-filarial effect and potentially decreases the number of required treatment rounds (3-7). This new triple therapy (IDA) was recently recommended by the WHO as the preferred regimen for LF elimination in some settings (8).

Although LF treatment is safe, transient mild to moderate systemic adverse events (AEs) are common following treatment, and these are especially common in individuals with circulating Mf (3). Furthermore, the risk of AEs and AE severity are positively
correlated with blood Mf counts (Mf/mL) (9). Systemic AEs are not direct effects of the drugs on the host, because they are quite uncommon in uninfected individuals (10). The pathogenesis of these AEs is not completely understood, but they are believed to be triggered by host responses to dying filarial worms. Post-treatment AEs have been associated with increases in plasma levels of IL-6, TNF-α and soluble TNF receptor (11, 12). We recently reported significant increases in 16 cytokines in persons who experienced AEs after treatment during a clinical trial that was performed in Papua New Guinea (13). These results were consistent with LPS-like stimulation of cytokines with increases in TNF-α, IL-1β, IL-6, IL-1RA and IL-10.

*Wolbachia* are intracellular α-proteobacteria that are present in filarial species that cause LF. The bacteria are hypothesized to trigger AEs when they are released by dying parasites after treatment. One study detected free *Wolbachia* DNA in blood collected 4-48 hours after LF treatment in individuals with moderate and severe AEs, but bacterial DNA was not detected in blood from most individuals with no or mild AEs (14). Some features of AEs are consistent with the effects of LPS. A filarial (*Brugia malayi*) antigen with LPS-like characteristics was described some years ago (15). However, the *B. malayi*-associated *Wolbachia* genome (16) does not include orthologues of genes responsible for the biosynthesis of lipid A (a component of LPS) (17). It is therefore unlikely that *B. malayi Wolbachia* contains LPS in its cell wall. Bioinformatic analysis of the *Wolbachia* genome predicts the presence of a *Wolbachia* lipoprotein: peptidoglycan-associated lipoprotein (PAL) (18). A synthetic, lipolated version of the N-terminus of *Wolbachia* PAL can signal through TLR2-TLR6 and induce pro-inflammatory responses *in vitro* in murine and human cells and *in vivo* in mice (18). Additionally, the diacylated
N-terminal polypeptide of the *Wolbachia* PAL (WoLP) was identified as the main trigger for a neutrophil inflammatory response through a TLR2-TLR6 dependent mechanism *in vivo* in human samples from individuals infected with *Onchocerca volvulus* (19). Recently PAL was confirmed by proteomics as one of the most abundant proteins in extracts from adult *B. malayi* female worms(20).

Besides *Wolbachia*, post-treatment AEs could also be triggered by immune complexes (IC) that develop after treatment of LF. ICs are aggregated antigens, antibodies, and components of the complement cascade that can activate pro-inflammatory pathways (21). It has been reported that filarial antigen levels increase with a concurrent decrease in filarial specific antibodies post-treatment, and these changes were temporally associated with the development of AEs, suggesting that AEs might be caused by IC (22). Circulating IC (CIC) have also been shown to increase post-treatment (23), and CIC precipitated from LF-infected individuals can activate granulocytes to release pro-inflammatory cytokines (24). CIC activate the classical complement pathway.

AEs are common after treatment for LF, and fear of AEs reduces population compliance with MDA (25). Therefore, the goal of this study was to improve understanding of the pathogenesis of AEs after LF treatment. We hypothesized that AEs are caused when filarial worm components are released after treatment and interact with the host innate or adaptive immune systems and that this would be associated with specific biomarker and gene expression profiles. To test this hypothesis we measured filarial antigen, CIC, LPS Binding Protein (LBP) and components of the complement cascade in plasma before and after treatment, and we studied host transcriptional
responses and cytokine profiles in LF-infected individuals who experienced AEs after treatment.

3.3 Methods.

Study design and sample collection.

Buffy coat and plasma samples were collected during an open label filariasis treatment study in the Agboville District in southeastern Côte d’Ivoire (Clinicaltrials.gov NCT # 02974049). Written informed consent was obtained from all participants. Adults with *W. bancrofti* microfilaremia were randomly assigned to one of four treatment arms (all oral medications): the standard LF treatment regimen for Côte d’Ivoire (200µg/kg IVM plus 400mg ALB), IDA: 200µg/kg IVM plus 6mg/kg DEC and 400mg ALB, a single 400 mg dose of ALB, or a single 800 mg dose of ALB. A subset of ninety-five individuals treated with either IVM/ALB, IDA or 400mg ALB had samples processed for use in the AE study described in this paper. We selected these individuals based on the availability of pre- and post-treatment samples and clinical AE data. Metadata of these 95 individuals is shown in Supplemental Table 1.

A physical examination was performed shortly before treatment, and vital signs were recorded. A review of systems (ROS) questionnaire was also completed to assess subjective symptoms prior to treatment. Venous blood (3 to 4 mL in EDTA) was collected immediately before participants received treatment. Participants were interviewed and examined the next day to assess AEs, and venous blood was collected approximately 24 hours after treatment. Blood samples were centrifuged within an hour of collection, and plasma was removed. The buffy coat (approximately 500µL) was
carefully aspirated with a pipette and added to 1.8mL of RNAlater (Ambion, Foster City, CA). The plasma samples and buffy coat/RNAlater samples were stored at the study site at -20°C, shipped frozen, and later stored at -80°C.

**Adverse event classification.**

AEs were categorized as mild, or moderate. Those with moderate AEs (n=9) had at least two new or worsening subjective symptoms plus one objectively measured change in their vital signs (an increase in axillary temperature of ≥ 0.8°C to at least 37.4°C post-treatment and/or a decrease in sitting systolic blood pressure of at least 20 mm Hg). Individuals with subjective or objective AEs that did not fulfill the criteria for moderate AEs were considered to have had mild AEs (n=24). Individuals with no new objective or subjective symptoms after treatment were considered to have no AEs (n=62).

**Circulating filarial antigen assay.**

A direct sandwich enzyme immunoassay (EIA) was performed as previously described (13). This assay uses the monoclonal antibody AD12 that binds to a carbohydrate epitope on circulating filarial antigen (CFA). It is important to note that the carbohydrate epitope recognized by AD12 is present in many filarial glycoproteins (26). However, the high molecular weight CFA is the only filarial antigen that is frequently detected in the blood of *W. bancrofti*-infected individuals. Pre- and post-treatment plasma samples from 95 individuals were tested in duplicate. The detection range of the CFA EIA was 6.3 to 400 ng/mL. CFA was detected in all samples, but two individuals had extremely high CFA levels that were above the upper detection limit of the assay. These
samples were retested after dilution to obtain baseline CFA concentrations. The percent change in CFA relative to baseline following treatment was calculated for each participant. Sample pairs with pre-treatment values less than 20 ng/mL (7 individuals) were excluded from the percent calculations, because they were near the lower detection limit of the assay. Kruskal-Wallis H tests were used to compare percent change values and absolute values between the three AE groups and the three treatment arms. Wilcoxon signed-rank tests were used to compare pre- and post-treatment CFA levels within the three AE groups.

**Immunoprecipitation and Western blot.**

Nine paired (pre- and post-treatment) samples with high CFA levels at baseline were selected for this analysis; six of these participants had moderate AEs, one had mild AEs, and two had no AEs. 15mg of a monoclonal antibody (DH6.5) that detects the same carbohydrate epitope as AD12 was directly conjugated to 2mL of agarose Affigel 10 beads (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. Conjugated beads were stored as a 50% solution in PBS. 40µL of conjugated beads were mixed with 50µl of human plasma and 300µl PBS and rocked overnight at 4°C. The beads were washed four times with cold PBS and then boiled in 1X NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) to release bound antigens. Proteins were resolved by SDS-PAGE using a 4-12% bis-tris NuPAGE gradient gel (Invitrogen) and transferred to 0.45µM nitrocellulose membrane (Amersham, Piscataway, NJ). Membranes were blocked with 5% milk in phosphate buffered saline with tween-20 (PBS-T) followed by incubation with a peroxidase-conjugated AD12 antibody (1:3000 dilution) for one hour at
room temperature. After washing, membranes were incubated with Clarity Western ECL substrate (Bio-Rad). Chemiluminescence was detected by a ChemiDoc imager (Bio-Rad), and results were analyzed using Image Lab 5.2.1 software.

**Immune complex assay.**

CIC were measured with a C1q ELISA. C1q was purchased (Sigma-Aldrich, St. Louis, MO), and a previously published protocol was followed (13). Plasma samples were available from 41 individuals for this assay (8 with moderate AEs, and 33 with no AEs), and both pre- and post-treatment samples were tested in duplicate. Negative control samples (plasma samples from healthy North American subjects and deionized water) were tested on each plate. Values were expressed as µg/mL of AHG (aggregated human gamma globulin) (Invitrogen). The range of detection for the CIC ELISA was 0.0006 to 6 µg/mL of AHG, and all samples had detectable CIC. Mann-Whitney U tests were used to compare absolute CIC levels between the two AE groups pre- and post-treatment. The Wilcoxon signed-rank test was used to compare post-treatment CIC levels to baseline levels within AE groups. The Kruskal-Wallis H test was used to compare absolute CIC levels between the three treatment arms post-treatment.

**Complement component assays.**

Nine individuals with moderate AEs were matched to individuals with no AEs following treatment. Matching was based on age, sex, baseline Mf count, and treatment arm (Supplemental Table 2). Complement component 3 (C3), complement component 4 (C4) and Factor B (FB) were measured in the 36 samples (18 matched case-control
subjects pre- and post-treatment) with ELISA kits (AssayPro, St. Charles, MO). The C3 and C4 assays were competitive enzyme immunoassays, and the FB assay was a sandwich ELISA. Each sample was tested in duplicate and manufacturer’s protocol was followed. Paired t-tests were used to compare pre- and post-treatment complement component levels by AE group.

**LPS binding protein assay.**

LBP was measured with a sandwich ELISA kit (Abnova, Taipei, Taiwan). Plasma samples from the same 18 matched case-control subjects were included. Each sample was tested in duplicate and manufacturer’s protocol was followed. Paired t-tests were used to compare pre- and post-treatment levels within both AE groups. The range of detection for the LBP ELISA was 5 to 50 ng/mL.

**Cytokine assays.**

Twenty-seven cytokines (IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin-1, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF) were measured with the MAGPIX system with the Bio-Plex Human 27-Plex Cytokine Panel and Bio-Plex Cytokine Reagent Kit (Bio-Rad). Plasma samples from the same 18 matched-control subjects were included. A previous paper includes the detailed protocol (13). Briefly, all samples were tested in duplicate, standard curves were calculated using the manufacturer’s software, and the analysis considered mean concentrations (pg/mL) from two duplicate wells. Wilcoxon signed-rank tests were used to determine whether
cytokine levels changed after treatment in either of the two AE groups. Mann-Whitney U tests were used to determine whether pre- or post-treatment cytokine levels were different between the two AE groups. For graphing, fold changes were calculated for each cytokine by AE groups, and samples with cytokines below the detection limit were assigned a value equal to half the value of the lowest pre-treatment sample concentration measured for that cytokine.

**RNA preparation.**

RNA was extracted from pre- and post-treatment buffy coat samples from the same 18 matched case-control subjects. Total RNA was extracted using Qiagen RNeasy kits (Qiagen, Hilden, Germany) according to the manufacturer’s protocol with an added homogenization step and on-column DNase digestion as follows. For each sample 200μL of the buffy coat/RNAlater mixture was added to 700μL of the kit’s RLT buffer and vortexed. The mixture was then added to a QIAShredder column (Qiagen) and centrifuged for 2 min at 16,000g. The flow-through was added to 700μL of 70% ethanol, and this mixture was added to a RNeasy column. Bound RNA was eluted in 30μL RNase-free water and stored at -80°C. The quality and quantity of RNA was verified with a Bioanalyzer 2100 (Agilent Technologies, Cedar Creek, Texas). Samples were processed with the TruSeq Stranded Total RNA LT Sample Prep Kit with Ribo-Depletion using the manufacturer’s protocol (Illumina, San Diego, CA). The RNA was high quality (average RIN value 9.3, range 8.5-10).
**RNA sequencing and mapping.**

The 36 samples were sequenced in two batches. The first 14 samples were sequenced with the HiSeq2000 (2x 100 PE run and Illumina TruSeq Stranded Total RNA) platform, and the remaining 22 samples were sequenced with HiSeq4000 (2x 150 PE run and Illumina TruSeq Stranded Total RNA). Between 28-41 million read fragments per sample were mapped to 19,864 protein-coding genes. Raw reads were mapped to protein coding genes using HISAT2 (version 2.0.5) (27), and the human reference genome GRCh38.84. FeatureCounts (28) was used to count reads per gene.

**Differential gene expression and overall expression patterns.**

DESeq2 (29) was used to generate normalized read counts and to identify differentially expressed genes between the different comparator groups (namely AEs vs. no AEs and pre- vs. post-treatment). The program “R” with the biocLite package “DESeq2” was used. Gene expression results from individuals before and after treatment were considered to be repeated measures for the analysis. Principal component analysis (PCA) was performed for 500 genes with the greatest variability in expression (based on DESeq2 output, default settings), and distance metrics statistics (30) were used to determine whether groupings affected overall expression patterns. A clustering dendrogram (Euclidean distance, complete linkage) was also used to illustrate overall expression patterns, and this method considered all genes. A two-tailed binomial distribution with unequal variance (for categorical data), and Mann-Whitney U tests (for continuous variables) were used to identify over-represented metadata variables in the different clustering groups such as baseline Mf/mL and treatment group.
Functional enrichment in the post-treatment AE group.

The online tool WebGestalt (31) was used to identify enriched KEGG pathways within genes that were upregulated post-treatment during AEs. The reference set was a list of all 19,864 genes with expression signals in the RNAseq data, and the default values were used except the significance level (FDR < 0.05). The program i-cisTarget (32) was used to identify enriched transcription factor binding sites in the upregulated gene set using default settings and database version 4.0.

Identification of similar expression profiles.

GeneQuery is an online tool that can search the PubMed GEO database and compare transcriptional signatures to published gene expression profiles (33). The input for the post-treatment AE profile were 744 genes that were identified by differential gene expression (DESeq2) to be upregulated post-treatment in individuals with moderate AEs.

Changes in peripheral blood leukocyte populations after treatment

CIBERSORT(34) is an analytical tool that can estimate the abundances of 22 leucocyte subtypes based on RNA-seq data. Pre- and post-treatment DESeq2 normalized read counts were used as input for the program. The standard LM22 (22 immune cell types) was the signature gene file, and all default settings were used. Thirteen cell subtypes had very low representation in this dataset (totaling less than 4% in all 36 samples), so the analysis was limited to the remaining subtypes. Percent change for each
cell type post-treatment was calculated for the two AE groups, and Mann-Whitney U tests were used to assess the significance of differences by AE group.

**Prioritization of the genes with altered regulation post-treatment in individuals with AEs.**

Random forest (RF) analysis was used to prioritize the 678 genes that were differentially expressed in individuals with moderate post-treatment AEs. RF was performed using the “R” package “randomForest” with 1000 trees and default values to analyse DESeq2 normalized read counts. Differentially expressed genes were ranked based on decreasing Mean Decrease in Accuracy values for 10 separate RF models. The Mean Decrease in Accuracy is the decrease in model accuracy from permuting the values in each feature. This metric is used to compare the impact of the variables in the model, and a large positive value indicates that a variable was closely linked to AE group across the dataset.

**Preparation of cDNA for validation of selected differentially expressed genes.**

Additional RNA was extracted from residual buffy coat samples that were available for 34 of the 36 samples that were subjected to expression profiling (17 individuals pre- and post-treatment) as described above. Extracted RNA was treated with DNase I (Invitrogen), and RNA was measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). Each sample was diluted to approximately 0.5ng/µL RNA with RNase-free water. cDNA was prepared with SuperScript II Reverse
Transcriptase (Invitrogen) and with Oligo(dT)_{12-18} according to the manufacturer’s protocol.

**Validation of the top differentially expressed genes by quantitative reverse-transcription PCR (qRT-PCR).**

SYBR Green based assays were performed for the top eight genes based on the RF analysis (*DIP2B, ZCCHC6, RBPJ, PELI1, FNDC3B, TLR2, LTBR, NT5C2*) that were upregulated in peripheral blood leukocytes (PBL) after treatment in participants who experienced moderate AEs. Four housekeeping genes (*SDHA, ACTB, HPRT1* and *YWHAZ*) were used as controls for these experiments. We chose these based on prior validation as housekeeping genes by others (35) and because our results confirmed their stable gene expression before and after treatment. Pre-validated primer sets for the eight target genes were purchased from KiCqStart SYBR Green Primers (Sigma-Aldrich), and primers for the four housekeeping genes were made using previously published sequences (IDT, Coralville, IA) (Supplemental Table 3). Real-time PCR reactions were performed with 10µL of SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 450 nmol/L of each primer, and 2µL cDNA (approx. 1ng RNA) with a final volume of 20µL. Thermal cycling was performed for 40 cycles with a QuantStudio 7-Plex Real-Time PCR System (Applied Biosystems), and cycle threshold (Ct) values were determined using the manufacturer’s software. All samples were tested in duplicate, and each plate included a negative water control and a RNA sample that had not been treated with reverse transcriptase. Delta delta Ct values were calculated (36), using the geometric mean Ct value of three housekeeping genes (*SDHA, ACTB* and *YWHAZ*) as a
normalization factor (35). Student’s t-tests were performed to compare baseline and post-treatment delta Ct values by AE group.

**Statistical methods.**

All statistical analyses were performed with IBM SPSS (version 23). Shapiro-Wilk tests were used to test for normality in each sample set, and additional tests were performed as described in each section above. Logistic regression analysis was performed with the binary dependent variable AEs (moderate AEs vs. no AEs). The independent variables considered included age, sex, treatment arm, baseline Mf/mL, and baseline CFA level.

Separate RF analyses of gene expression and plasma biomarker data were performed 10 times using 1000 trees. The output was the average Mean Decrease in Accuracy over the 10 runs for each variable.

**Ethical review.**

Institutional review boards in Cleveland, USA (University Hospitals Cleveland Medical Center IRB #08-14-13) and in Côte d’Ivoire (Comité National d’Ethique et de la Recherche, CNER, N: 008/MSLS/CNER/-kp) approved the clinical trial study protocol. Written informed consent was received from all participants prior to inclusion in the study.
3.4 Results.

Study population.

This study of the pathogenesis of AEs that occur after LF treatment used human samples that were obtained as part of a clinical trial for LF that was conducted in Côte d’Ivoire. Full results from that study have not yet been published, but early results from the study have been reported in published abstracts (4, 5). Briefly, 189 *W. bancrofti*-infected adults were randomly assigned to one of four treatment arms as described above, and all participants had AE assessments performed 24 hr after treatment. The AE study enrolled a subset of 95 treated participants. Supplemental Table 1 summarizes the specific analyses that were performed on samples from each of the 95 individuals. Nine of these participants experienced moderate AEs (Table 1), 24 had mild AEs, and 62 had no AEs. There was no difference in age or sex distribution between the three AE groups (Supplemental Table 4).

Multiple additional filarial antigens were detected in post-treatment plasma samples.

Baseline CFA levels were positively correlated with baseline Mf counts (Spearman’s rho: 0.51, *P* < 0.001), and absolute CFA levels were significantly higher at baseline in individuals who developed moderate AEs compared to individuals who developed mild or no AEs after treatment (*P* = 0.012 by Kruskal-Wallis H test). Plasma CFA levels increased post-treatment in all three AE groups, but the increases were greater in persons with moderate AEs (*P* < 0.05 by Kruskal-Wallis H test) (Figure 1A). Percent changes in CFA levels post-treatment were significantly lower in the individuals
treated with only ALB compared to those in individuals treated with IVM/ALB or IDA ($P < 0.0001$ by Kruskal-Wallis H test).

Western blot analysis was performed for nine pre- and post-treatment plasma pairs to compare CFA patterns detected in plasma before and after treatment. All nine pretreatment plasma samples contained only a single high molecular weight parasite antigen as expected, and this antigen was also present in post-treatment plasma samples. However, four of the post-treatment plasma samples contained many parasite antigens that were not present before treatment. Two examples are shown in Figure 1B (P1 had moderate AEs, and P2 had mild AEs), and this pattern was also observed in plasma from two other participants who experienced moderate AEs following treatment. Western blot results obtained with five other post-treatment plasma samples tested (3 from persons with moderate AEs, and 2 from persons with no AEs) were no different from those observed in pre-treatment samples.

**CIC did not increase post-treatment and the classical complement pathway was not activated in individuals with moderate AEs.**

All pre- and post-treatment samples contained CIC. However, there was no difference in CIC levels between the two AE groups before or after treatment, and CIC levels did not significantly change after treatment in either AE group (Supplemental Figure 1). There was also no difference in post-treatment CIC levels between the treatment arms.

C3 levels significantly decreased post-treatment in individual with moderate AEs, but this change was not observed in individuals with no AEs (Figure 2A). C4 levels did
not change in either AE group (Figure 2B). Factor B levels decreased post-treatment in most individuals with moderate AEs, but three individuals had increases in FB levels, and the group differences were not significant (Figure 2C).

**LPS binding protein levels increased post-treatment in individuals with moderate AEs.**

LBP was detected in all pre- and post-treatment samples. LBP levels increased post-treatment in individuals with moderate AEs ($P = 0.0007$ by paired t-test), but they did not increase in individuals with no AEs (Figure 2D).

**Many plasma cytokines increased in plasma after treatment in persons who experienced moderate AEs.**

Plasma cytokine levels before and after treatment are shown by AE group in Figure 3. Seven cytokines (IL-8, MCP-1, VEGF, TNF-α, MIP-1β, G-CSF and IFN-γ) increased post-treatment only in individuals who experienced moderate AEs ($P < 0.05$ by Wilcoxon signed-rank test). Five cytokines (IL-6, IL-10, IL-1RA, IP-10 and MIP-1α) increased post-treatment in individuals with and without AEs ($P < 0.05$ by Wilcoxon signed-rank test), but three of these (IL-6, IL-10 and IL-1RA) had significantly higher levels post-treatment in individuals with moderate AEs compared to individuals with no AEs ($P < 0.05$ by Mann-Whitney U tests) (Figure 3). The remaining 15 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin-1, GM-CSF, PDGF-BB and RANTES) did not change after treatment in either AE group. There was no difference in pre-treatment cytokine levels between individuals that would
develop moderate AEs and individuals that would not develop AEs for any of the 27 cytokines.

**Changes in gene expression associated with moderate post-treatment AEs.**

Raw and processed RNA-seq data are available to the public on NCBI’s Gene Expression Omnibus (Accession number: GSE110146).

We analyzed changes in gene expression in PBL after treatment to further elucidate host responses associated with AEs. Post-treatment gene expression profiles from persons who developed moderate AEs clustered together using a clustering dendrogram based on gene expression profiles across all genes (Figure 4A). Post-treatment AE samples were significantly overrepresented in the fourth group (bolded in Figure 4A, $P$-value $< 0.0001$ for enrichment within the cluster, two-tailed binominal distribution with unequal variance). Higher levels of baseline Mf/mL were also observed in this group ($P$-value = 0.038, Mann-Whitney U test), but none of the other metadata categories (treatment arm or village) were over-represented. Age also did not affect clustering. A similar pattern was observed by principal components analysis (Figure 4B), where post-treatment moderate AE samples clustered together and were clearly separated from their pre-treatment controls ($P$-value = 0.005 by PERMANOVA (30)). No other differences were significant between the four groups by PCA.

We used differential gene expression analysis to identify the genes that were responsible for the clustering of the post-treatment moderate AE samples. At a very stringent significance threshold ($P < 10^{-5}$ according to DESeq2 output), 783 genes were identified to be upregulated after treatment ($n$=744) or before treatment ($n$=39) in
individuals who experienced moderate AEs (Figure 4C). No differences were observed pre- or post-treatment in individuals with no AEs when this stringent significance threshold was used. However, at a less stringent $P$-value of 0.05, there were 126 genes upregulated post-treatment and 19 genes upregulated pre-treatment in individuals without AEs (Figure 4D). There was only one overlapping gene in the genes upregulated pre-treatment in individuals with and without AEs, whereas the majority of the genes upregulated post-treatment in individuals with no AEs were also upregulated post-treatment in individuals with AEs (Figure 4E).

We then assessed whether there was evidence for functional enrichment in the genes upregulated post-treatment in individuals with AEs. Among the 744 upregulated genes post-treatment in the AE samples a total of 35 enriched biological pathways (KEGG) were identified (Supplemental Table 5), and these included TLR signaling and downstream pathways such as NF-κB, TNF and Jak/STAT. Many individual genes in the TLR signaling pathway, including $\text{TLR2, TLR6, STAT1 and STAT2}$, were identified by DESeq2 to be significantly upregulated post-treatment in individuals with AEs. A separate analysis (i-cisTarget) predicted that six transcription factors were over-represented in the differentially expressed genes (Supplemental Table 6), and three of these, $\text{STAT1, STAT2 and IRF1}$, are downstream of TLR signaling. Convincingly, $\text{STAT1}$ and $\text{STAT2}$ were therefore identified by two independent analyses, signifying the importance of these two transcription factors in the development of AEs. IRF1 is activated by IFN-γ and is a major transcription factor of IL-8, and correspondingly both IFN-γ and IL-8 levels significantly increased post-treatment in individuals with AEs. The complete TLR signaling pathway highlighting the individual upregulated genes, KEGG
pathways and transcription factors, was constructed with the use of the online database SPIKE (37) (Supplemental Figure 2). Finally we wanted to compare our newly identified LF AE transcriptional signature to published gene expression profiles. The post-treatment AE transcriptional signature of the 744 upregulated genes was very similar to multiple published endotoxin exposure gene expression profiles, in addition to many other profiles (Supplemental Table 7).

After successfully identifying a significant transcriptional signature of post-treatment AEs, we explored whether a pre-treatment transcriptional signature could predict what individuals would go on to develop AEs after treatment. There were no significant differentially expressed genes at baseline between individuals that would develop moderate AEs, and individuals that would not develop AEs (by DESeq2).

**Neutrophils increased and lymphocytes decreased post-treatment.**

Changing cell populations can have a large effect on gene expression profiles. Differential cell counts were unavailable, so cell type proportions were estimated using the RNA-seq data. This analysis suggested that neutrophils increased more and lymphocytes decreased more post-treatment in individuals with AEs compared to individuals with no AEs (Fig. 5A). These changes are consistent with stress-type immune responses. For simplicity, B cells (memory and naïve), T cells (CD8, CD4 naïve and memory resting) and NK cells were combined into one category (lymphocytes) in Figure 5A, and ungrouped data are presented in Supplemental Figure 3. Estimated leucocyte proportions at baseline were very similar between the two AE groups, but individuals
who experienced post-treatment AEs had significantly fewer estimated memory B cells compared to individuals who did not develop AEs after treatment (Figure 5B).

**Prioritization of genes upregulated post-treatment in individuals with AEs show that TLR2 is one of the most important genes for the development of AEs.**

The genes upregulated post-treatment in individuals with AEs were prioritized by importance for AE development using a machine-learning tool (RF analysis). This was done in order to identify genes with the strongest associations between expression levels and development of AEs and to identify genes of interest for PCR validation. Table 2 shows the top 15 genes that were upregulated in persons who developed moderate AEs after treatment. However, based on this analysis it was not possible to determine whether the gene expression changes were the cause or effect of the AEs that were experienced.

**Orthogonal validation of expression levels for candidate genes confirmed RNA-seq data.**

qRT-PCR studies were performed to confirm whether expression of genes identified by DESeq2 and RF analyses was actually increased post-treatment in individuals with moderate AEs. Increased expression after treatment was confirmed for seven of the top eight genes (*DIP2B, ZCCHC6, PELI1, FNDC3B, TLR2, LTBR* and *NT5C2*) (Figure 6). Expression of the eighth gene (*RBPJ*) did not change after treatment in either AE group. Expression of the housekeeping gene *HPRT1*, did not change with treatment in either AE group (as expected).
Modeling identified high baseline CFA levels as predictor for development of post-treatment AEs and post-treatment increases in LBP levels as another risk factor.

We were unable to identify any pre-treatment transcriptional signature that could predict moderate AEs. We therefore wanted to assess if any metadata or baseline infection parameter was associated with the development of moderate AEs. A logistical regression was performed to consider effects of age, sex, treatment arm, baseline Mf/mL and baseline CFA on the risk for development of post-treatment moderate AEs. A total of 71 individuals were included in the model (9 moderate AEs and 62 no AEs). The logistic regression model was statistically significant, $X^2 (6) = 22.1, P = 0.0012$. The model explained 50.2% (Nagelkerke $R^2$) of the variance in AE outcome, and correctly predicted 93% of outcomes. However the model was better at predicting people who did not develop AEs; it correctly predicted only 44.4% of the individuals who developed moderate AEs. Increasing baseline CFA levels were associated with increased likelihood of developing AEs ($P = 0.022$), but the other independent variables did not significantly contribute to the model. RF analysis was performed on the same dataset (71 individuals), and this also identified the baseline CFA level as the best predictor for subsequent development of AEs. However, treatment arm was also a positive predictor in the RF model, and could therefore be related with the development of AEs (Table 3). It was surprising that baseline Mf count was not identified by the logistic regression model or RF to significantly contribute to correctly predicting AEs. However, baseline Mf counts were higher in individuals who developed moderate AEs (geometric mean 343 Mf/mL) compared to individuals with no AEs (geometric mean 188 Mf/mL), and this difference was significant ($P = 0.036$ by Mann-Whitney U test). RF analysis was also used to
identify the variable (CFA, CIC, C3, C4, FB or LBP) that was best at classifying AE outcome based on post-treatment fold change in the 18 matched case-control subjects. LBP changes after treatment was the only variable that was significantly associated with the development of AEs (Supplemental Table 8).

3.5 Discussion.

This study looked at changes in proteins in plasma and changes in gene expression in PBL in persons who experienced moderate AEs following treatment of LF.


Filarial antigen levels increased in plasma after treatment in individuals with moderate AEs, and this agreed with our recently published results from a separate clinical trial (13). Western blot results from this study showed that many new filarial antigens with the carbohydrate epitope detected by the monoclonal antibody AD12 appeared in the blood 24 hours after treatment in some individuals. In contrast, only a single high molecular weight antigen circulates in the blood of *W. bancrofti*-infected individuals without treatment (26). We postulate that treatment kills or injures worms so that they release internal antigens that are normally concealed inside the parasite.

Results from this study also confirmed our previous finding that plasma CIC levels do not increase after treatment of LF in persons who develop moderate AEs (13). This finding suggests that AEs after LF treatment are not caused by CIC. The complement cascade (classical pathway) modulates pro-inflammatory effects of CIC (38). Activation of the classical complement cascade leads to decreased C3 and C4,
whereas activation of the alternative pathway (AP) leads to decreased C3 and factor B (FB). Our results are most consistent with activation of the AP by parasite antigens, as C3 and FB decreased in individuals with moderate AEs while there was no change in C4 levels. The RNA-seq data also supports the AP hypothesis, because expression of CFP (complement factor properdin- a positive regulator and initiator of the AP) significantly increased post-treatment in individuals with moderate AEs (adjusted P-value 0.001, DESeq2). In contrast, expression of C4B (basic form of C4, part of the classical pathway) significantly decreased (adjusted P-value 0.04, DESeq2) post-treatment in individuals with moderate AEs. Additionally, IFN-γ and TNF-α are known to induce FB synthesis (39). This could account for the inconsistent FB levels post-treatment in individuals with moderate AEs, because both IFN-γ and TNF-α increased in these individuals; the positive stimulus of these cytokines may have counteracted decreases in FB levels as it was used in the AP. In summary, many different filarial antigens were transiently released post-treatment, but they did not appear to form CIC or activate the classical complement cascade.

PBL appeared to respond to the release of filarial antigens by releasing cytokines, and the cytokine profiles of post-treatment AEs in LF infected individuals are complex. In our previous study of samples from a treatment trial in Papua New Guinea, we reported that 16 cytokines increased post-treatment in individuals with moderate AEs (13), and eight of these cytokines were also increased after treatment in this study (IL-1RA, IL-6, IL-10, G-CSF, MCP-1, MIP-1β, TNF-α, and VEGF). It is not surprising that more cytokines increased in the previous study, because more time-points were sampled. Also, participants in the Papua New Guinea study had higher blood Mf counts and higher
rates and severity of AEs than participants in the present study, and this may account for their stronger cytokine responses. A new finding in this study was the increase in IFN-γ post-treatment in individuals with moderate AEs. This was supported by the fact that IRF1 (downstream of IFN-γ) was identified to be an important transcription factor for AE development. The increase in IL-8 levels paralleled the increase in neutrophils post-treatment in individuals with moderate AEs. Results from this study did not confirm the finding from the prior Papua New Guinea study that high levels of eotaxin-1 pre-treatment are a risk factor for development of post-treatment AEs. Again this discrepancy may be related to differences in infection intensity between participants in the two studies. Levels of IL-6 and TNF-α have previously been shown to positively correlate to levels of Wolbachia DNA in human plasma 48 hours after treatment of LF (40).

**Changes in gene expression in PBL in persons who experienced moderate AEs after treatment.**

RNA-seq was performed to better understand changes in leukocyte gene expression that occur in persons who experienced moderate AEs after treatment. We identified a distinctive transcriptional signature associated post-treatment moderate AEs with 783 genes that were differentially expressed (at the $P < 10^{-5}$ level) in persons who experienced moderate AEs. In contrast, no gene was differentially expressed at that level of significance before or after treatment in individuals who did not experience AEs. 95% of the 783 genes associated with AEs exhibited increased expression post-treatment. Thus, moderate AEs were primarily associated with upregulation of gene expression and not with gene suppression. A total of 126 genes were upregulated post-treatment in
individuals with no AEs at the low stringency $P < 0.05$ level, but 83% of these genes were also upregulated post-treatment in individuals with moderate AEs. Thus changes in gene expression after treatment did not always lead to clinically evident AEs.

The transcriptional signature results are consistent with the hypothesis that *Wolbachia* lipoprotein activates TLR2-TLR6 (18, 41), as bacterial lipoproteins can induce pro-inflammatory responses through TLR2 signaling and NF-κB and STAT1 activation (42). The finding that *TLR2* was one of the genes most highly associated with the development of moderate AEs also supports this hypothesis. Furthermore, LBP was found to increase post-treatment in plasma from individuals with moderate AEs, and RF analysis identified LBP (fold change post-treatment) as the best variable for classifying AE outcome. LBP is an acute-phase protein that is mostly known for its function of shuttling LPS to TLR4 via CD14. However, it can also shuttle lipoproteins to TLR2 also via CD14 (43) as would be the case with PAL. CD14 expression was upregulated post-treatment in individuals with moderate AEs, and it had one of the most significant adjusted $P$-values ($6.4e^{-26}$) in the dataset. CD36 is another accessory receptor for the TLR2-TLR6 heterodimer (44), and this gene was also upregulated post-treatment in individuals with moderate AEs (adjusted $P$-value 0.004). The LF AE transcriptional signature was similar to previously published endotoxin exposure gene expression profiles further supporting the *Wolbachia* lipoprotein hypothesis. Since multiple filarial antigens that are normally not accessible to the immune system are released post-treatment, it is possible that *Wolbachia* components are released into the host’s circulation in a similar fashion. Thus we cannot be certain that *Wolbachia* lipoprotein is the only or prime trigger for AEs. It is possible that other filarial components can signal
through TLRs and contribute to the development of AEs, and this might explain why expression of many other TLRs (TLR1, TLR4, TLR5, TLR6 and TLR8) were upregulated post-treatment in individuals with moderate AEs. Many different ligands can activate TLR2, including protozoan ligands such as GPI anchors from *Trypanosoma cruzi* (45) and *Leishmania major* (46). *Wolbachia*-independent activation of the immune system causing severe AEs is seen in *Loa loa* (a filarial worm that lacks *Wolbachia* (1)) infected individuals post-treatment suggesting that *Wolbachia* is not the sole cause of AEs after anti-filarial treatment. TLR signaling is clearly associated with the development of AEs, but complement activation has similar downstream effects, and there is considerable crosstalk between these two pathways (47). Thus both TLR signaling and the complement AP could be actively involved in the pathogenesis of AEs. Another possible mechanism for AEs following treatment of LF is that treatment abrogates the normally dominant Th2 immune responses stimulated by helminth infections that interfere with the expression and function of TLRs (48). Increased TLR expression and signaling after treatment may then induce pro-inflammatory Th1 responses causing AEs. Indeed, classic Th1 cytokines TNF-α and IFN-γ were increased after treatment in people with moderate AEs.

We did not detect a pre-treatment transcriptional signature that was a significant risk factor for development of post-treatment AEs. Baseline CFA levels were the best predictor for moderate AEs in this study, and this was the only variable that was a significant predictor by logistic regression. CFA levels are correlated with Mf counts and likely with adult worm numbers, and this result suggests that CFA levels are related to
the total parasite biomass that can potentially contribute to the development of AEs after treatment.

**Genes upregulated post-treatment in individuals with AEs.**

An important finding from this study was that post-treatment AEs in LF-infected individuals are associated with upregulation of hundreds of genes. A prioritized list of the top 15 genes important for the development of AEs is listed above in Table 2. These genes and their associated pathways may provide insight into the pathogenesis of AEs. In addition to the TLR pathway, Notch, NF-κB and IL-1 signaling were common themes. Four of the top 15 genes (RBPJ, TLR2, ALDH1A2 and APLP2) are involved in TLR/Notch signaling. The Notch pathway is involved in development and is conserved from *Drosophila* to mammals. RBPJ is involved in the crosstalk between TLR and Notch signaling that is thought to help fine-tune the immune response through negative and/or positive feedback (Supplemental Figure 4) (49). NF-κB is another downstream pathway of Notch signaling, and three of the top 15 genes (PELI1, TLR2 and LTBR) are involved in NF-κB signaling. TLR2 is a receptor for the canonical NF-κB pathway, and PELI1 is involved in intracellular downstream signaling. The canonical pathway results in the release of pro-inflammatory cytokines, such as IL-6 and TNF-α. Thus, activation of this pathway is consistent with the cytokine profiles individuals who experienced moderate AEs after treatment. Interestingly, lymphotoxin beta receptor (LTBR) stimulation has been shown to enhance the LPS-induced expression of IL-8 via the combined action of NF-κB and IRF1 (50), and this is consistent with our results. Finally, IL-1 is clearly associated with the development of AEs. *IL-1RAP* was one of the top 15 genes identified
by RF, and this gene is one of two co-receptors for IL-1. Additionally, expression of the second co-receptor IL-1RI was increased post-treatment in individuals with AEs. Both expression (this study) and plasma levels (our prior study (13)) of IL-1β increased after treatment in individuals with AEs. Inhibitors of the IL-1 pathway were also upregulated post-treatment in individuals with AEs. This included both increased expression and protein levels of IL-1RA and increased expression of the IL-1β decoy receptor IL-1R2. These results illustrate the importance of the balance between the pro-inflammatory effects of IL-1β and the anti-inflammatory effects of IL-1RA for AE development.

Limitations of the study.

One limitation of this study was that the RNA-seq was performed on mixed PBL samples. This makes it difficult to separate the effects of altered gene expression from the effects of changing cell type proportions. Separating different types of leukocytes was not feasible, because the samples were collected in rural Côte d’Ivoire and processed in a simple field lab. On the other hand, this study provides insight into the pathogenesis of post-treatment AEs. Additionally, it was possible to estimate the different cell subtypes present in PBLs using the RNA-seq data, so we could associate changes in gene expression with altered cell types to decrease the chance that the former was a directly result of the latter. For example, if the post-treatment AE transcriptional signature had been similar to a neutrophil gene expression profile it could have been caused by the increasing proportion of neutrophils post-treatment and not due to specific neutrophil activation during AEs. Another limitation was that we did not study an untreated control group, because it would have been unethical to withhold treatment from infected
individuals. For the cytokine analysis we did not correct for multiple comparisons, however, the results are generally consistent with our previous findings (13), and this increases our confidence in the results. Based on the standard significance level of 0.05, approximately 3 differences would be expected to be significant by chance for 54 tests (27 cytokines measured pre- and post-treatment in two AE groups), whereas 17 comparisons were significant at the 0.05 level in this study. The semi-quantitative Filariasis Test Strip (FTS) was used to assay CFA in the field, whereas a quantitative ELISA was used to measure CFA levels in the laboratory setting in this study. Newer studies have demonstrated that tests that detect LF CFA (including FTS) can cross-react with *L. loa* antigens that circulate in blood from a subset of individuals with heavy infections (51), and with biological samples from animals infected with *L. loa* and *Onchocerca ochengi* (52, 53). This cross-reactivity was not a concern for this study because *L. loa* is not endemic to Côte d’Ivoire, and the area of Côte d’Ivoire where the study was conducted is non-endemic for *O. volvulus*.

In future studies it would be interesting to measure *Wolbachia* DNA in pre and post-treatment samples from individuals with AEs after treatment of LF and onchocerciasis to try to correlate bacterial DNA release with host expression profiles post-treatment. *Wolbachia* DNA has been shown to increase post-treatment in individuals with AEs after treatment of LF (14). Additionally, peak *Wolbachia* DNA levels have been shown to be correlated with AE reaction scores in individuals treated with DEC or IVM for onchocerciasis (54).
Conclusions.

This study included first global RNA-seq analysis of PBLs from LF-infected individuals, and it has provided novel insights into the pathogenesis of a clinically relevant problem. The samples were ideal for studying AEs after LF treatment, because each post-treatment sample was paired with a pre-treatment sample from the same individual. This internal control improved our ability to study the AE phenotype in humans. AEs represent a significant challenge for the global program to eliminate LF, and the fear of AEs in communities receiving MDA is a main factor that reduces compliance (25). Minimizing the impact of AEs has therefore been identified as a key component for successful MDA programs (25). More than 850 million individuals have been treated as part of GPELF, and a significant percentage of these individuals experience AEs.

This study has also provided a framework for investigating the host responses associated with severe AEs that occur after treatment of other filarial worms such as *O. volvulus* and *L. loa*. Treatment of other, more familiar infections can also result in severe AEs that are caused by host responses to dying pathogens. This Jarisch-Herxheimer reaction occurs after antibiotic treatment of spirochetal infections such as syphilis, Lyme disease, leptospirosis, and relapsing fever, and it is also hypothesized to be caused by the release of bacterial lipoproteins that activate TLR2 (55). The transcriptomic response during the Jarisch-Herxheimer reaction has not been studied, so the dataset from this study could provide a valuable starting point for research on this related clinical problem.

To recap our major findings, this study has provided new insights regarding the pathogenesis of post-treatment AEs in LF-infected individuals. Our results are consistent
with the hypothesis that a *Wolbachia* lipoprotein triggers AEs by binding to TLR2-TLR6, but other uncharacterized filarial antigens might also play a role. Since TLR, NF-κB, and TNF pathways are involved, these pathways could potentially be targeted to prevent or treat AEs after LF treatment. We also found that high pre-treatment CFA levels were the best predictors of post-treatment AEs. This finding could be relevant for treatment-naïve areas with high LF infection prevalence and intensities. Individuals with high CFA levels pre-treatment (assessed with the FTS (56)) could be offered non-steroidal anti-inflammatory medications together with antifilarial medications for home management of moderate or severe AEs. However, a positive FTS from an individual who resides in or has traveled to an area that is also endemic for *L. loa* needs to be interpreted with caution due to the issues of cross-reactivity mentioned above. Information from this study should allow program managers and drug distributors to reassure populations and communicate to them that AEs experienced after LF treatment are transient and caused by host responses to dying or injured parasites.

### 3.6 Acknowledgements.

The authors would like to thank the technicians, physicians and study participants in Agboville, Côte d’Ivoire, for their support during sample and data collection. All authors report no conflict of interest.
3.7 References.


Figure 3.1: Filarial antigens increase post-treatment in individuals with moderate adverse events (AEs).

Circulating filarial antigen (CFA) levels increased significantly more post-treatment in individuals who experienced moderate adverse events (mod AEs) compared to individuals with no AEs \( (P < 0.05 \text{ by Kruskal-Wallis}) \) \( (n=62 \text{ with no AEs, } n=24 \text{ with mild AEs, } n=9 \text{ with moderate AEs}) \). Boxes indicate the interquartile range \( (25^{\text{th}} \text{ and } 75^{\text{th}} \text{ percentile of data distribution}) \), and horizontal lines within the boxes are median values. The whiskers show 95% confidence intervals around the median values.
Figure 3.2: Levels of complement components and lipopolysaccharide binding protein (LBP) pre- and post-treatment (n=9 with moderate adverse events (AEs), n=9 with no AEs).

[A] Complement component 3 (C3) significantly decreased post-treatment in individuals with moderate AEs (*P = 0.03 by paired t-test). [B] Complement component 4 (C4) did not change with treatment in either AE group. [C] Complement Factor B (FB) did not change with treatment in either AE group. [D] LBP levels significantly increased post-treatment in individuals with moderate AEs (***P = 0.00007 by paired t-test).
Levels of IL-8, MCP-1, VEGF, TNF-α, MIP-1β, G-CSF and IFN-γ significantly increased post-treatment in individuals with moderate AEs, and levels of IL-1RA, IL-6, IL-10, MIP-1α and IP-10 increased significantly post-treatment in individuals with moderate AEs and in individuals with no AEs (*P < 0.05, **P < 0.001 by Wilcoxon signed-rank tests). IL-1RA, IL-6 and IL-10 increased more in individuals with moderate AEs (*P < 0.05 by Mann-Whitney U tests).
Figure 3.4: Overall expression patterns and enrichment pre- and post-treatment (n=9 with moderate adverse events (AEs), n=9 with no AEs).

[A] Sample clustering (Euclidean distance) based on gene expression profiles across all genes. Post-treatment AEs (red) samples are significantly overrepresented in the fourth group (bolded, \( P < 0.0001 \), binomial distribution). Treatment arms; A: albendazole (ALB), IA: ivermectin (IVM) and ALB, IDA: IVM, diethylcarbamazine and ALB. [B] Principal component analysis of paired samples. Post-treatment AEs samples (red) are significantly different from their pre-treatment controls (yellow) (\( P = 0.005 \) by PERMANOVA). No other differences between the four groups are significant. [C]/[D] Expression plots comparing pre- and post-treatment gene expression in individuals with moderate AEs [C] and individuals with no AEs [D]. Red dots represent genes significantly upregulated post-treatment, and blue dots are genes significantly upregulated pre-treatment (\( P < 10^{-5} \) for the AE group [C], and \( P < 0.05 \) for the no AE group [D] from DESeq2). [E] Overlap between the genes upregulated pre- and post-treatment between the two AE groups.
Figure 3.5: Estimated leucocyte subtypes (CIBERSORT) for the two adverse events (AEs) groups.

[A] Percent change post-treatment of leukocyte subtypes. Estimated lymphocytes decreased more and estimated neutrophils increased more post-treatment in individuals with moderate AEs (n=9) compared to individuals with no AEs (n=9). [B] Estimated leukocyte proportions pre-treatment. Individuals that did not develop AEs (n=9) had higher levels of estimated memory B cells pre-treatment compared to individuals that developed moderate AEs (n=9). *P < 0.05 by Mann-Whitney U tests.
Figure 3.6: qRT-PCR validation of RNA-seq data.

Post-treatment fold change of the top eight genes identified by random forest analysis. Seven of the genes (DIP2B, ZCCHC6, PELI1, FNDC3B, TLR2, LTBR and NT5C2) significantly increase only in individuals with moderate adverse events (AEs) (n=8) compared to individuals with no AEs (n=9). *P < 0.05, **P < 0.001 by t-tests. HPRT1 is a housekeeping gene that should not change with treatment in either AE group.
Table 3.1: Clinical characteristics of the nine individuals with moderate adverse events (AEs) after LF treatment.

<table>
<thead>
<tr>
<th>ID</th>
<th>Treatment arm</th>
<th>Sex</th>
<th>Age</th>
<th>Mf/mL(^A)</th>
<th>Objective AEs(^B) (Pre and Post)(^C)</th>
<th>Subjective AEs(^D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>IVM/ALB</td>
<td>M</td>
<td>35</td>
<td>349</td>
<td>37.0 - 38.5</td>
<td>HA(1), fever(1)</td>
</tr>
<tr>
<td>6</td>
<td>IVM/ALB</td>
<td>M</td>
<td>60</td>
<td>700</td>
<td>120/70 - 100/60</td>
<td>HA(1), N/V(1)</td>
</tr>
<tr>
<td>7</td>
<td>IVM/DEC/ALB</td>
<td>F</td>
<td>32</td>
<td>264</td>
<td>35.7 - 38.0</td>
<td>Fever(2), joint pain(1)</td>
</tr>
<tr>
<td>8</td>
<td>IVM/DEC/ALB</td>
<td>M</td>
<td>48</td>
<td>660</td>
<td>36.7 - 38.2</td>
<td>HA(1), fever(1), rash(1), cough(1)</td>
</tr>
<tr>
<td>9</td>
<td>IVM/DEC/ALB</td>
<td>M</td>
<td>52</td>
<td>229</td>
<td>36.3 - 37.4</td>
<td>HA(2), fever(2), rash(1), joint pain(1), fatigue(1)</td>
</tr>
<tr>
<td>10</td>
<td>IVM/DEC/ALB</td>
<td>M</td>
<td>34</td>
<td>308</td>
<td>37.0 - 37.8</td>
<td>HA(1), fever(1), joint pain(1), muscle pain(1), fatigue(1)</td>
</tr>
<tr>
<td>11</td>
<td>IVM/ALB</td>
<td>M</td>
<td>29</td>
<td>560</td>
<td>37.5 - 38.5</td>
<td>Fever(1), rash(1), muscle pain(1)</td>
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<tr>
<td>12</td>
<td>IVM/DEC/ALB</td>
<td>M</td>
<td>69</td>
<td>503</td>
<td>170/90 - 120/80</td>
<td>N/V(1), rash(2), dark urine(1), cough(1), joint pain(1)</td>
</tr>
<tr>
<td>17</td>
<td>IVM/DEC/ALB</td>
<td>M</td>
<td>32</td>
<td>79</td>
<td>37.0 - 37.9</td>
<td>N/V(2), dyspnea(2)</td>
</tr>
</tbody>
</table>

\(^A\)Mf/mL: microfilaria per mL of blood. \(^B\)Objective AEs: increase of at least 0.8°C to at least 37.4°C post-treatment (axillary temperature), or a decrease of at least 20mmHg systolic pressure post-treatment. \(^C\)Pre- and post-treatment temperatures or blood pressures are shown. \(^D\)Subjective AEs: symptoms that the patient reported during the review of systems questions. Grades: 1; mild symptom and patient could attend work or school. 2; patient could not attend school/work. HA: headache, N/V: nausea/vomiting.
Table 3.2: Top 15 genes associated with development of moderate adverse events in LF patients after treatment as identified by random forest analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Description</th>
<th>Mean Decrease in Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIP2B</td>
<td>Disco interacting protein 2 homolog B</td>
<td>3.30</td>
</tr>
<tr>
<td>ZCCHC6</td>
<td>Zinc finger CCHC-type containing 6</td>
<td>3.18</td>
</tr>
<tr>
<td>RBPJ</td>
<td>Recombination signal binding protein for Ig kappa J region</td>
<td>3.07</td>
</tr>
<tr>
<td>PEL11</td>
<td>Pelino E3 ubiquitin protein ligase 1</td>
<td>2.99</td>
</tr>
<tr>
<td>FNDC3B</td>
<td>Fibronectin type III domain containing 3B</td>
<td>2.95</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll like receptor 2</td>
<td>2.79</td>
</tr>
<tr>
<td>LTBR</td>
<td>Lymphotoxin beta receptor</td>
<td>2.65</td>
</tr>
<tr>
<td>NT5C2</td>
<td>5'-nucleotidase, cytosolic II</td>
<td>2.57</td>
</tr>
<tr>
<td>KIAA1551</td>
<td>KIAA1551</td>
<td>2.32</td>
</tr>
<tr>
<td>ALDH1A2</td>
<td>Aldehyde dehydrogenase 1 family member A2</td>
<td>2.08</td>
</tr>
<tr>
<td>ZSWIM6</td>
<td>Zinc finger SWIM-type containing 6</td>
<td>1.97</td>
</tr>
<tr>
<td>APLP2</td>
<td>Amyloid beta precursor like protein 2</td>
<td>1.95</td>
</tr>
<tr>
<td>MYLIP</td>
<td>Myosin regulatory light chain interacting protein</td>
<td>1.95</td>
</tr>
<tr>
<td>FGD4</td>
<td>FYVE, RhoGEF and PH domain containing 4</td>
<td>1.92</td>
</tr>
<tr>
<td>IL1RAP</td>
<td>Interleukin 1 receptor accessory protein like 2</td>
<td>1.90</td>
</tr>
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</table>
Table 3.3: Logistic regression and random forest analysis of sex, age, treatment arm and baseline infection parameters on the development of moderate adverse events.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Logistic Regression Model</th>
<th>Random Forest</th>
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<tbody>
<tr>
<td></td>
<td>$B$</td>
<td>Odds ratio</td>
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<tr>
<td>Constant</td>
<td>-4.79</td>
<td>-</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.24</td>
<td>0.79</td>
</tr>
<tr>
<td>Age</td>
<td>0.03</td>
<td>1.03</td>
</tr>
<tr>
<td>Treatment arm$^\text{A}$</td>
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<td></td>
</tr>
<tr>
<td>IDA</td>
<td>0.54</td>
<td>1.72</td>
</tr>
<tr>
<td>ALB</td>
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<tr>
<td>Mf/mL</td>
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<tr>
<td>CFA</td>
<td>0.02</td>
<td>2.69$^\text{B}$</td>
</tr>
</tbody>
</table>

$^\text{A}$The coefficients for treatment arms are contrasts with the standard treatment, ivermectin (IVM) plus albendazole (ALB). IDA: IVM, diethylcarbamazine (DEC) and ALB. $^\text{B}$Reported odds ratio for CFA (circulating filarial antigen) is for an increase of 50 ng/mL, because the range of CFA concentrations was wide (7-700 ng/mL).
Supplemental Figure 3.1: Circulating immune complex levels.

Mean circulating immune complex (CIC) levels ± standard error pre and post-treatment in individuals with no adverse events (AEs) (n=33) and individuals with moderate AEs (n=8). There was no significant difference between pre- and post-treatment values within the two AE groups (Wilcoxon signed-rank test), or between the two AE groups (Mann-Whitney U tests). AHG: aggregated human gamma globulin.
**Supplemental Figure 3.2:** Overview of TLR signaling.

TLR signaling pathway with highlighted upregulated genes in black (based on DESeq2 analysis) and upregulated KEGG pathways (based on WebGestalt analysis) in red. Overrepresented transcription factors (based on i-CisTarget analysis) are in blue.
**Supplemental Figure 3.3:** Estimated leukocyte subtypes post-treatment.

Percent change post-treatment of leukocyte subtypes in people with and without adverse events (AEs). T cells (CD4 naïve) decrease more and neutrophils increase more post-treatment in individuals with AEs (n=9) compared to individuals with no AEs (n=9). *P < 0.05, **P < 0.01 by Mann-Whitney U tests.
Supplemental Figure 3.4: TLR and Notch.

Crosstalk between the TLR signaling pathway and the Notch pathway. Expression and/or function of various components of the Notch pathways could be regulated by TLR signaling. Conversely, Notch pathway components positively or negatively modulate TLR-activated transcriptional, translational, and metabolic programs to finetune outcomes of immune responses. Figure and text from Shang Y, Smith S, and Hu X. Role of Notch signaling in regulating innate immunity and inflammation in health and disease. *Protein Cell* 2016;7(3):159-74.
**Supplemental Table 3.1:** Metadata for all 95 individuals including which samples were used for all the experimental tests.

<table>
<thead>
<tr>
<th>Study ID</th>
<th>Treatment arma</th>
<th>Age</th>
<th>Sex</th>
<th>Mf/mL</th>
<th>AEa group</th>
<th>CFA ElA</th>
<th>Western Blot</th>
<th>CIC elisa</th>
<th>C3/C4 and FB</th>
<th>LBP</th>
<th>Cytokines</th>
<th>RNA-seq</th>
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<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
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**A** Treatment arm: 1=ALB/IVM, 2=ALB, 4=ALB/IVM/DEC

**B** AE group: 0=no AEs, 1=mild AEs, 2=moderate AEs
Supplemental Table 3.2: Characteristics of each adverse event (AE) case and matched control with no AEs.

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### Supplemental Table 3.3: Primer sequences.

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**Supplemental Table 3.4:** Age and sex distribution in the three adverse events (AEs) groups.

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<sup>A</sup>Kruskal-Wallis H test  
<sup>B</sup>Chi-squared test
Supplemental Table 3.5: Upregulated KEGG pathways post-treatment in individuals with adverse events (WebGestalt analysis).

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</table>

^Kegg pathway name. C: the number of reference genes in the category. O: the number of genes in the gene set and also in the category. E: the expected number in the category. R: ratio of enrichment. P-value: P-value from hypergeometric test. FDR: P-value adjusted by the multiple test adjustment.
**Supplemental Table 3.6:** Enriched transcription factor binding sites in the 744 genes upregulated post-treatment in individuals with adverse events.

<table>
<thead>
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<th>Transcription factor binding site</th>
<th>Normalized enrichment score</th>
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<td>STAT2</td>
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<td>SPI1</td>
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<td>STAT3</td>
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<tr>
<td>IRF1</td>
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<td>CEBPB</td>
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**Supplemental Table 3.7:** Upregulated KEGG pathways pre-treatment in individuals that did not develop adverse events (Gene Set Enrichment Analysis).

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<th>NAME</th>
<th>SIZE</th>
<th>ES</th>
<th>NES</th>
<th>NOM P-val</th>
<th>FDR q-val</th>
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</table>

\(^{A}\text{KEGG pathway. Size: Number of genes in the gene set after filtering out those genes not in the expression dataset. ES: enrichment score for the gene set; that is, the degree to which this gene}\)
set is overrepresented at the top or bottom of the ranked list of genes in the expression dataset. NES: normalized enrichment score; that is, the enrichment score for the gene set after it has been normalized across analyzed gene sets. NOM P-val: Nominal P-value; that is, the statistical significance of the enrichment score. The nominal P-value is not adjusted for gene set size or multiple hypothesis testing; therefore, it is of limited use in comparing gene sets. FDR q-val; false discovery rate; that is, the estimated probability that the normalized enrichment score represents a false positive finding. FWER P-val: Familywise-error rate; that is, a more conservatively estimated probability that the normalized enrichment score represents a false positive finding.
**Supplemental Table 3.8:** Variables in the random forest model (post-treatment fold change).

<table>
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<tr>
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<th>Average Mean Decrease in Accuracy</th>
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<tr>
<td>Complement Factor B (FB)</td>
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<tr>
<td>Complement component 4 (C4)</td>
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</tbody>
</table>
Chapter 4:

Discussion, future directions and conclusion
Preface

This chapter was written by BJA. Comments from GJW and PUF were incorporated into the final version, presented here.
4.1 Comparison of the two sample cohorts: Papua New Guinea and Côte d'Ivoire.

Researchers and clinician scientists have been interested in understanding the adverse events (AEs) that occur after lymphatic filariasis (LF) treatment for decades, but the pathogenesis has been difficult to define. We decided to study AEs using human samples that were collected in different LF treatment trials in Papua New Guinea and Côte d'Ivoire. The main findings from the Papua New Guinea pilot study (1) showed that various filarial components such as antigen and DNA are released post-treatment in individuals with AEs. Contrary to our hypothesis and a previously published paper (2), we did not observe an increase in circulating immune complexes (CICs) post-treatment in individuals with AEs. Additionally, we found that the cytokine response during AEs is complex with a large number of cytokines being released after treatment. The cytokine response observed in individuals with AEs was similar to the cytokine response after endotoxin exposure, and was therefore consistent with Wolbachia exposure. However, based on this study we were unable to determine what filarial or Wolbachia components were responsible for the immune activation, and if the host response was caused by phagocytosis of filarial/Wolbachia components, by direct interaction between foreign antigens and host cell surface receptors or by a combination of both. In order to delineate the immune response during AEs, we decided to look at the host gene expression before and after treatment in leucocytes from individuals with and without AEs.

In order to address this question we took leverage of a clinical trial conducted in Côte d'Ivoire (Clinicaltrials.gov NCT # 02974049) to collect plasma and buffy coat samples. The RNA-seq analysis of RNA extracted from pre- and post-treatment buffy coat samples identified a significant transcriptional signature of post-treatment AEs in
LF-infected individuals. TLR and NF-κB signaling were overrepresented, and overall the gene expression profile was consistent with the hypothesis that the Wolbachia lipoprotein, PAL, signals through TLR2 (3). We also established that LPS Binding Protein (LBP) increases more post-treatment in individuals with AEs, and this finding also supports the Wolbachia/TLR2 signaling pathway. These new findings are presented schematically in Figure 4.1A. We confirmed the majority of the cytokine results from the Papua New Guinea pilot study. Additionally, we found that the classical complement pathway does not appear to be activated during AEs, whereas the alternative complement pathway is possibly involved in the development of AEs. This was supported by the fact that we again did not observe an increase in CIC post-treatment in individuals with AEs.

We assessed Wolbachia DNA levels in plasma by qPCR, but obtained results that were contradictory to published literature. Previously published studies used nested PCR to measure Wolbachia DNA in human plasma (4), but we feel that this method is prone to contamination when using field samples, so we did not attempt this approach.

The Papua New Guinea study cohort was small with a total 24 enrolled participants, but it was a pharmacokinetic study so blood samples were available from many time points from pre-treatment up until 72 hours post-treatment (5). This repeated-sampling from the same individuals was helpful to examine the kinetics of cytokine release, and to assess when filarial components were detectable in the host blood. We found that 24 hours post-treatment was a good time-point to measure cytokines and filarial components because many cytokines peaked at around this time and CFA levels were increased. Additionally, AEs peak at around 24-48 hours post-treatment (6-10). For the larger Côte d'Ivoire trial we therefore decided to collect samples pre-treatment and at
24 hours post-treatment. The Papua New Guinea pharmacokinetic trial was conducted in 2014, and a collaborator had collected the blood samples. Our AE study was not the main focus of the pharmacokinetic trial, and this meant that we only had access to a very limited volume of plasma, and buffy coat samples had not been collected at all. For the larger clinical trial in Côte d'Ivoire I was in the field during pre-screening and for over one month during the enrollment period. I collected samples and taught the team how to process the blood to separate the buffy coat from the plasma. I also organized the clinical data assessment (vital signs, review of systems, and AE data) for each individual, and made a database with all this information. This effort resulted in paired pre- and post-treatment samples from over 120 treated individuals, and detailed clinical information for each person. This dissertation was unique in the sense that I was in a position to be involved in all the stages of the project; from pre-screening potential study subjects and preparing for the clinical trial, to enrolling and treating infected individuals in an endemic country, and finally conducting the bioinformatics analysis and additional laboratory experiments using the human samples that had been collected. Very few individuals in the Côte d'Ivoire trial developed moderate AEs. We selected a subset of participants to include in our study, and ended up with a cohort of 95 individuals including nine individuals with moderate AEs, 24 with mild AEs and 62 with no AEs. The AE rate was much higher in the Papua New Guinea cohort where out of the 24 participants 7 developed moderate AEs, 12 developed mild AEs and 5 had no AEs (5). This drastic difference in AE rate and severity was likely due to the fact that the Papua New Guinea individuals had much higher baseline levels of Mf compared to the individuals from the Côte d'Ivoire cohort.
4.2 Circulating immune complexes and the classical complement pathway are not involved in the development of AEs.

One of the hypotheses going into the project was that CIC formed after treatment could trigger AEs, however, we did not see an increase in CIC in the pilot study or the larger clinical trial study. This was surprising because a previous study had concluded that CIC increase post-treatment (2). An explanation for this discrepancy could be that CIC were measured much later after treatment in the previous study (7 days post-treatment), whereas we measured CIC at 24 hours post-treatment in the Côte d'Ivoire cohort and up until 72 hours post-treatment in the Papua New Guinea cohort. It takes time for the immune system to generate antibodies, so perhaps CIC form later post-treatment, but it would be difficult to attribute AEs that peak at 24 hours post-treatment to CIC that are formed multiple days after treatment. A 12-day course of DEC treatment was used in the previously published study, whereas we only treated participants once. The longer treatment regimen could also have led to more CIC formation.

On the other hand, the CIC results agree with the complement data, because the classical complement cascade does not appear to be involved in the development of AEs, because C4 levels did not change post-treatment. CIC are part of the initial stage of the classical pathway, and can bind to the C1-complex to activate the pathway (11). Whereas the classical complement pathway recognizes and is activated by antigen-antibody complexes, the alternative pathway is much less stringent in its recognition requirements. In the alternative pathway spontaneous hydrolysis of C3 allows C3b to covalently attach to a wide range of substrates, including endotoxin and bacterial polysaccharides (12). The alternative complement cascade is not known to be activated by bacterial lipoproteins,
such as *Wolbachia* PAL. However, one could easily imagine that as the parasites die and disintegrate post-treatment, a subset of the many released filarial and *Wolbachia* antigens could act as substrates for the promiscuous C3b, and thereby activate alternative complement pathway.

4.3 **An allergy-like host response does not appear to be involved in the development of AEs.**

It is well established that prominent eosinophilia and elevated serum IgE levels are common features of human LF infections (13). It is therefore conceivable that an allergy-type host response could be involved in the development of post-treatment AEs, because infected individuals are poised for such an immune reaction. Mast cells, basophils and eosinophils are essential effector cells in allergic inflammation (14). In order to monitor for an allergic response we measured histamine, a main effector of basophils and mast cells, and four different eosinophil granule proteins. Histamine was measured in 18 individuals (9 with moderate AEs, and 9 with no AEs) pre-treatment and 24 hours post-treatment from the Côte d'Ivoire study cohort. Unfortunately only two samples had histamine levels over the detection limit of the assay, and both of those samples were pre-treatment. Four eosinophil granule proteins were measured: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO). For the eosinophil granule assays four individuals from the Papua New Guinea cohort were selected (two with moderate AEs and two with no AEs) and samples from 13 time-points from pre-treatment until 72 hours post-treatment were tested. The samples were shipped to the Nutman laboratory at the
NIH where the assays were performed. MBP, ECP and EDN were detected in all samples, whereas EPO was undetectable in all the samples. With this very limited sample set no differences were observed between the two AE groups, and levels of MBP, ECP and EDN did not change much after treatment (Dr. Thomas Nutman, personal communication). In onchocerciasis eosinophil granule proteins are released after eosinophils attach to the Mf by an adherence reaction (15). *O. volvulus* Mf reside in the skin (16), and it is unlikely that a similar eosinophil reaction occurs in the blood stream where *W. bancrofti* Mf are found.

For the two main clinical trials described in this thesis; the pharmacokinetic study in Papua New Guinea and the larger clinical trial in Côte d'Ivoire, we were unable to complete white blood cell differential analysis for the enrolled subjects. However, in 2016 our group conducted a pharmacokinetic study in Côte d'Ivoire (Clinicaltrials.gov NCT # 02845713) (17), as briefly described in chapter 3. For this study we performed white blood cell differentials for all enrolled subjects pre-treatment and at 24 and 48 hours post-treatment. We did not observe any significant change in eosinophil numbers post-treatment, and there was no significant difference in eosinophil counts/percentages pre- or post-treatment between individuals with and without AEs. Half of the individuals in the pharmacokinetic study were uninfected, so we were able to confirm that at baseline individuals infected with LF have significantly higher eosinophil counts (mean approximately 600 eosinophils/uL) compared to uninfected individuals (mean approximately 300 eosinophils/uL). Based on this limited data we are unable to confirm or reject the hypothesis that a type IV immune response is involved in the development of post-treatment AEs in LF-infected individuals.
4.4 Current hypothesis: a range of filarial and Wolbachia antigens are released after treatment, and these activate the host immune system resulting in AEs.

We have shown that multiple filarial components, including a range of antigens and DNA, are released within hours after treatment, and that individuals with AEs have higher levels of these parasite components in their blood. We have also shown that serum levels of many cytokines increase after treatment, and most of these only increased in individuals with AEs. This cytokine profile is complex with some cytokines peaking within just hours after treatment (IL-12 and IL-17A); others only increase days after treatment (IL-7) and some remain elevated for an extended period of time (IL-6, IL-10, MIP-1β and IL-8). Finally, we have assessed the pathogenesis of host responses during AEs by examining gene expression profiles of leucocytes. The most obvious functional enrichments post-treatment in individuals with AEs were the TLR and NF-κB signaling pathways. This included upregulation of six different TLR genes (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR8), signifying the diverse nature of possible immuno-stimulatory signals post-treatment. This is because each TLR recognizes specific ligands: triacylated lipoproteins (TLR1-TLR2), diacylated lipoproteins (TLR2-TLR6), LPS (TLR4), flagellin (TLR5), and ssRNA (TLR8) (18). Furthermore, many additional biological pathways were upregulated (Supplemental Table 3.2). For example, both phagocytic and the NOD-like receptor signaling pathways were upregulated post-treatment in individuals with AEs, indicating that phagocytosis and downstream intracellular recognition of foreign antigens are associated with AE development. Natural killer cell mediated cytotoxicity and apoptosis are also both upregulated post-treatment in individuals with AEs. This
broad immune activation suggests that there are many different activators of the host response, and that no single antigen is responsible for AE development.

It is reasonable to suspect that *Wolbachia* is one of the main causes of AEs in *W. bancrofti*-infected individuals, because we know that bacteria can induce strong pro-inflammatory host responses. However, compared to the vast body of literature concerning bacterial molecules, virulence factors and toxins and the resulting pro-inflammatory host responses, very little is known about how filarial nematodes can activate the immune system. This is turn might result in overestimation of *Wolbachia* involvement in AE development. Previous studies have concluded that live filarial Mf interact with dendritic cells and cause an upregulation of mRNA expression of pro-inflammatory molecules such as IL-8, RANTES, IL-1α, TNF-α, and IL-β (19). This immune activation could be the results of secreted filarial proteins. It is also possible that uncharacterized filarial components can signal through TLRs and contribute to the development of AEs. For example, various protozoan ligands can signal through TLR2, including GPI anchors from *Trypanosoma cruzi* (20), *Leishmania major* (21), *Plasmodium falciparum* and *Toxoplasma gondii* (22), in addition to a *T. cruzi*-released protein (23). Uncharacterized filarial proteins could be released by injured Mf and and/or adult parasites after treatment and activate the host response. This hypothesis is presented schematically in Figure 4.1B.

During infection the filarial parasites manipulate the host immune system and induce a modified Th2 response in addition to a muted Th1 response, and this allows the parasites to evade host defenses (24). Filarial parasites also down-modulate expression and function of TLRs and MHC receptors (19, 25-27). These immunomodulatory effects
are active parasite responses that require energy (e.g., to produce and secrete immunomodulatory molecules). After treatment the parasites are injured or dead, and vulnerable to attack from the host immune cells. These damaged parasites are no longer able to manipulate the host immune response. IVM has even been shown to inhibit the release of immunomodulatory molecules from the parasites (28). In onchocerciasis IVM treatment resolves the parasite-induced immunosuppression and restores the immune system (29, 30). Instead of the controlled release of specific immunomodulatory molecules, a whole range of filarial antigens that are not normally accessible to the host immune system are released into the circulation, and some of these could interact with TLR receptors. Furthermore, a subset of the released foreign antigens could act as substrates for C3b and activate the alternative complement pathway. This shift from a chronic and controlled Th2 dominated response to an acute Th1 response may contribute to development of AEs.

4.5 The pathogenesis of the Jarisch-Herxheimer reaction is similar to the pathogenesis of post-treatment AEs in LF-infected individuals.

A phenomenon similar to the post-treatment AEs observed in LF-infected individuals is common after treatment of more familiar infections. This phenomenon where dead or dying microbes release foreign antigens after treatment that trigger pro-inflammatory responses and result in AEs is known as the Jarisch-Herxheimer reaction. This reaction occurs after antibiotic treatment of spirochetal infections such as syphilis, Lyme disease, leptospirosis, and relapsing fever. These infections are present world-wide, and depending on the pathogen and the treatment course, a large percentage of
individuals experience the Jarisch-Herxheimer reaction post-treatment (31). Unlike the AEs experienced after LF-treatment, the Jarisch-Herxheimer reaction can be fatal with the highest risk in pregnant women and in neonates (32-34). There are many similarities between the AEs described in this thesis and the Jarisch-Herxheimer reaction. Non-endotoxin antigens are also hypothesized to cause the Jarisch-Herxheimer reaction, and in Treponema pallidum a lipoprotein has been identified as the likely trigger for the reaction (35). In Borrelia burgdorferi, the outer surface protein A lipoprotein stimulates cells by activating TLR2-TLR6 and IL-1R signaling (36), and this is very similar to the transcriptional signature of post-treatment AEs covered in chapter 3. Cytokine responses during the Jarisch-Herxheimer reaction are also similar, with increases in TNF, IL-6, IL-8 and IL-10 (37, 38). Finally, it has also been suggested that the alternative complement pathway is activated during the Jarisch-Herxheimer reaction (39). However, there are also significant differences between the two post-treatment conditions. For example the symptoms of the Jarisch-Herxheimer reaction occur much sooner after treatment; in syphilis they start at four hours, peak at eight hours, and subside by 16 hours post-treatment (40), whereas in relapsing fever they starts at one-two hours, peak at four hours, and subside by eight hours post-treatment (41). Contrary, the AEs observed after LF-treatment peak at around 24-48 hours post-treatment (6-10). This is perhaps because many of the drugs used to treat these spirochetal infections are bactericidal (42), and could therefore be faster-acting than the LF drugs that interact with the host immune system to exert their anti-filarial effects (28, 43-45). Furthermore, bacteria may disintegrate much faster than nematode worms with their thick cuticle. The transcriptomic response during the Jarisch-Herxheimer reaction has not been studied, so
results from our filariasis study could provide a good starting point for studies of this very similar phenomenon. It is also possible that lessons learned from the Jarisch-Herxheimer field could be applied to LF-treatment. For example pre-treatment with anti-TNF antibodies has been shown to significantly decrease the rates of adverse reactions after treatment of relapsing fever (46, 47). However, using anti-TNF antibodies for treatment or prevention of post-treatment AEs in LF-infected individuals as part of the large MDA programs is not feasible, because the AEs are mostly mild to moderate and anti-TNF treatments are very expensive (48).

4.6 Multiple antigens that contain the AD12 epitope circulate in the blood after treatment.

In both the Papua New Guinea and Côte d'Ivoire cohorts we found that plasma levels of circulating filarial antigen (CFA) increased 24-48 hours post-treatment in individuals with AEs. Previous studies had described an increase in CFA levels 5-7 days post-treatment (49, 50), but it was not known that CFA levels increase so soon after treatment. This was also the first report of an association between post-treatment CFA levels and the development of AEs. In the Côte d'Ivoire study we additionally discovered that it was a whole range of different filarial proteins with the AD12 epitope that were released post-treatment, and these lower weight AD12 antigens were not present pre-treatment. This finding was surprising, because only the high molecular weight CFA had previously been detected in the blood of *W. bancrofti*-infected individuals (51, 52). Diagnostic tests for *W. bancrofti* that use the AD12 antibody against a carbohydrate epitope on CFA are widely used (53). The current test used by the Global Program to
Eliminate LF (GPELF) is the Alere Filariasis Test Strip (FTS) (54), but previously the immunochromatographic card test (ICT) (52) was used to detect this antigen. The new finding that multiple filarial antigens with the AD12 epitope are present in the blood post-treatment may help to explain the fact that some patients with heavy *L. loa* infections test positive for filarial CFA (55). Individuals infected with *L. loa* sometimes have exceptionally high parasite loads in their blood with Mf counts that can exceed 100,000 per mL (16). *L. loa* parasites cleared by the immune system may release crossreactive antigens that do not normally circulate in the blood of infected individuals. The carbohydrate epitope detected by the AD12 antibody is present on many nematode proteins and not limited to *W. bancrofti* (56), so it would not be surprising if this epitope were also present in antigens released when *L. loa* Mf die. Antigens immunoprecipitated with antibody AD12 (as described in section 3.5) from plasma of *L. loa* infected individuals with false positive FST/ICT tests produce a similar pattern by Western blot to antigens present in *W. bancrofti* post-treatment sera in Figure 3.5B (Dr. Philip Budge, personal communication). This finding has a potential impact on GPELF, because neither IVM nor DEC can be used safely in MDA campaigns in areas that are co-endemic for *W. bancrofti* and *L. loa* because of the risk of serious AEs in people with heavy *L. loa* infections (57, 58). Cross-reactivity of sera from people with *L. loa* infections in the filarial CFA test limits the value of this important diagnostic test in such areas. Fortunately, areas highly endemic for loiasis are restricted to a few countries in central Africa (59).
4.7 The search for a pre-treatment bio-marker associated with the development of post-treatment AEs.

One goal of this project was to delineate the pathogenesis of post-treatment AEs by studying the host response during AEs. However, during the course of the project we were interested to identify pre-treatment biomarkers or transcriptional profiles associated with the development of post-treatment AEs. Eotaxin-1 was a promising candidate identified in the Papua New Guinea pilot study, because baseline levels in individuals who would go on to develop moderate AEs after treatment were significantly higher than in individuals who had mild or no AEs (Figure 2.2). Unfortunately this result was not confirmed in the larger sample cohort from Côte d'Ivoire. A possible explanation for this disparity might be that the Papua New Guinea study participants had much higher baseline Mf levels, and the moderate AEs in this group were more severe than the moderate AEs in the Côte d'Ivoire study. All seven of the individuals in the moderate AE group in the Papua New Guinea study had temperatures > 38°C after treatment, whereas only three of nine individuals with moderate AEs in the Côte d'Ivoire study had temperatures > 38°C. The maximum temperature recorded in the Côte d'Ivoire study was 38.5°C, whereas the maximum temperature recorded in the Papua New Guinea study was 39.7°C, and four of the seven Papua New Guinea individuals with moderate AEs had temperatures > 39°C. However, the temperature data for the two studies are not fully comparable, because axillary temperatures were recorded in the Côte d'Ivoire study, and oral temperatures were recorded in the Papua New Guinea study. Thus, individuals in the moderate AE group in the Côte d'Ivoire study were probably similar to the individuals in the mild AE category in the Papua New Guinea study. Also, there was no significant
difference in baseline eotaxin-1 levels between the mild AE and the no AE groups in the Papua New Guinea study. Therefore, it is conceivable that eotaxin-1 might be a biomarker for AEs, but only for more severe presentations with high fevers. The bottom line here is that no cytokine was identified as a risk factor for developing AEs after treatment in the Cote d’Ivoire study.

Based on results from the multiple logistic regression model, the best pre-treatment predictor for AE development was baseline CFA level, and this variable was better at predicting AEs than the baseline Mf counts. Multiple previous studies have described the association between high baseline Mf loads and the development of AEs (49, 60). CFA is already a widely used marker of *W. bancrofti*-infection, and it is currently measured with the FTS diagnostic test. The FTS has many advantages over the microscopy required to visualize and quantify Mf in the blood. Advantages include the fact that day blood can be used for the FTS, whereas night blood has to be collected for Mf testing. Mf microscopy also requires trained personnel, electricity, and good quality equipment (61). The FTS is much easier and only takes 10-15 minutes to complete. The intensity of the test line in the FTS (scored visually or by densitometry) is correlated with filarial antigen levels as measured by ELISA (62). Thus FTS scores may be useful in the field for identifying individuals with increased risk of AEs after LF-treatment who might benefit from post-treatment paracetamol or ibuprofen.
4.8 Is there a pre-treatment transcriptional signature that is associated with the development of AEs after treatment of lymphatic filariasis?

This study was not specifically designed to identify a pre-treatment transcriptional signature that could predict AEs. We were not able to identify a single gene that was significantly predictive for development of post-treatment AEs. That would require many more samples than we had in this study. To get around this we used Gene Set Enrichment Analysis (GSEA) as described in section 3.5. With this method we were able to identify biological pathways that were overrepresented in the two pre-treatment AE groups. As discussed in chapter 3, pre-treatment samples from individuals who did not develop AEs were enriched for various B-cell pathways, and this finding was in agreement with the CIBERSORT data, that showed that memory B-cells were significantly higher pre-treatment in individuals who did not develop AEs. Additionally, these data were also consistent with the GeneQuery results that identified multiple B-cell signatures to be similar to the gene expression profile pre-treatment in people who did not develop AEs after treatment. This finding was surprising, and it is difficult to explain. Perhaps individuals with immune systems primed for humoral responses are protected from AEs. Another explanation could be that individuals with stronger humoral immune responses are better at clearing Mf, and as a result of lower Mf levels pre-treatment therefore experience fewer AEs after treatment. However, the RNA-seq data was obtained from nine matched-control subjects that did not significantly differ in baseline Mf levels between individuals that developed AEs and individuals that did not develop AEs. It is also difficult to understand how this information could be used by GPELF. It would be interesting to test whether AE rates following treatment of LF are decreased in
persons who have recently received vaccines that induce vigorous antibody responses to microbial antigens. It would be difficult to do this in practice, because vaccine programs usually target children and AEs following MDA for LF are more common in adults.

There was no clear pattern in the pathways overrepresented in pre-treatment samples from individuals who developed post-treatment AEs. However, two pathways identified by GSEA were associated with the cytochrome P450 pathway. Cytochrome P450 enzymes metabolize endogenous and exogenous chemicals including many drugs (63). This finding was surprising, because the two microfilaricidal drugs IVM and DEC do not have active metabolites that need cytochrome P450 to be activated. IVM is actually metabolized into inactive metabolites by cytochrome P450 (64), whereas DEC is metabolized independently of cytochrome P450 (65). ALB, on the other hand, is a prodrug, and it is metabolized by cytochrome P450 into its active metabolite albendazole sulfoxide (66). Albendazole sulfoxide is then further metabolized into the inactive albendazole sulfone by cytochrome P450 (66). However, both albendazole and albendazole sulfoxide have very limited short term effects on Mf or adult filarial parasites, and AE rates after ALB treatment in LF-infected individuals are very low (67). Consequently, increased pre-treatment levels of cytochrome P450 might result in faster breakdown of the AE-causing microfilaricidal drug, IVM. This might be expected to decrease drug levels, anti-filarial activity, and the rate and severity of AEs. Thus the observed GSEA results are unexpected and for now, unexplained. Additional studies will have to be conducted to verify whether there is any link between cytochrome P450 and post-treatment AEs in LF-infected individuals.
4.9 **Future directions.**

A possible future study would be to investigate AE-associated genes and cytokines in *in vivo* or *in vitro* models. Peripheral blood mononuclear cells (PBMCs) from LF-infected individuals could be stimulated with different filarial-worm components or whole filarial extract, and responses could be assessed by qRT-PCR and cytokine ELISAs. The main problem with this approach would be to obtain the PMBCs from LF-infected individuals, because it is very difficult to isolate and preserve PBMCs in endemic countries due to the lack of laboratory facilities in the remote areas where parasite densities and the risk of AEs are the highest. Another possible approach would be to develop an animal model for treatment-induced AEs in LF. The most appropriate model would be the *L. sigmondontis* mouse model (68, 69). An advantage of this model is that the mouse is a permissive host that develops an adaptive immune response to the parasite (70). Tools are readily available for measuring gene expression and cytokine levels in mice, and studies with various knock-out mice, such as TLR knock-outs, could be very informative. A disadvantage of this model is that the mice naturally clear the infection after 3-4 months (71). Also maintenance of the life cycle in the laboratory is challenging, because it requires both cotton rats (difficult to use in the USA) and mites (72). Another suitable model would be the *B. malayi*-gerbil model of infection (73). An advantage of this model is that *B. malayi* is one of the three filarial parasites that cause LF in humans, and it is very similar to the other two species (74). Additionally, the gerbil is also a permissive host for the *Wolbachia*-free filarial parasite *A. viteae* (75). By comparing post-treatment AE presentations in these two gerbil models, one could identify the contribution of *Wolbachia* to the development of AEs. The gerbil model is easier to
maintain in the laboratory than the *L. sigmodontis* mouse model, because *B. malayi* and *A. viteae*-infected gerbils can be purchased (76), and the whole lifecycle does not have to be maintained. A disadvantage of the gerbil model is the tools for genetic studies are limited compared to those for mice, and there are no knockouts available. Neither of these models has been used to study AEs after anti-filarial treatment, although it is known that infected gerbils experience AEs after DEC treatment (77).

This study represents the first RNA-seq analysis of LF-infected individuals, and we were therefore not able to answer all the important questions and complete all the relevant comparisons in this one study. An important comparison that needs to be completed is to compare gene expression profiles of LF-infected and uninfected individuals. This has previously been done with microarrays (78), as mentioned in the introduction. If we added uninfected individuals who were treated with ALB/IVM or IDA as a control group, this could help us to weed out genes that change expression following treatment in uninfected people. Samples for this type of study are already available, because pre- and post-treatment buffy coat samples were collected during the Côte d'Ivoire pharmacokinetic trial (Clinicaltrials.gov NCT # 02845713) (17) where *W. bancrofti*-infected and uninfected individuals were treated with IDA. It would also be interesting to look at gene expression and infection status (both antigen and Mf) from the individuals in our study at one and/or two years post-treatment. This would enable us to identify changes in gene expression that occur following successful treatment. One challenge with this idea is that most people treated with one or two doses of IDA or ALB/IVM do not completely clear the infection as they remain antigen positive even if they are Mf negative. Another interesting analysis would be do look at the gene
expression profiles at 24 hours post-treatment to identify any difference in host responses between the individuals that successfully cleared their Mf at one or two years post-treatment, and the individuals that had suboptimal treatment responses.

The methodology used for this project could be adapted to other neglected tropical diseases, including the two filarial parasites that cause the most severe post-treatment AEs: *O. volvulus* and *L. loa* (57, 58, 79). *L. loa* does not contain *Wolbachia*, so it would be very interesting to see if host responses during AEs in *L. loa*-infected individuals differ from those that occur in *W. bancrofti*-infected individuals. Serious AEs in *L. loa*-infected individuals often occur in the central nervous system, and such AEs are not seen after treatment *W. bancrofti*. However, it is not clear whether peripheral blood leukocytes would be informative for studies of CNS AEs following treatment of loiasis. The main AEs in *O. volvulus*-infected individuals are skin-related as the adult parasites live in the subcutaneous tissue and the Mf live in the upper dermis (16). For onchocerciasis it might therefore be interesting to collect skin snips of healthy tissue and from tissue affected by AEs. These samples could be from the same individual, and that could eliminate inter-individual differences and resulting noise in the RNA-seq data.

### 4.10 Conclusions.

Overall the rate of AEs in LF MDA programs might seem low, because AEs mainly occur in infected individuals. Additionally, one might argue that most AEs that do occur are mild and that they should not be of great concern for researchers or health officials. However, an astounding 6 billion LF MDA treatments have been delivered to hundreds of millions of individuals across the globe, and even mild or moderate AEs can
have a huge impact on the success of LF elimination programs based on MDA. This dissertation research has described changes in host gene expression and cytokine profiles associated with post-treatment AEs in LF-infected individuals, and the work has identified pathways that could be targeted to decrease or eliminate AEs, such as TLR2, NF-κB, TNF and the STAT transcription factors. TLR2, NF-κB, and STAT have all been identified as possible cancer drug targets (80-82), and anti-TNF antibodies are widely used to treat autoimmune disorders, so maybe developments in these other fields will benefit LF patients in the future. Widespread use of expensive molecularly targeted therapy is obviously not well suited for the huge global LF program, but such treatments might be used in the small number of persons who experience serious AEs. Our results are consistent with the hypothesis that *Wolbachia* lipoprotein activates TLR2-TLR6 resulting in AEs, but additional uncharacterized filarial antigens are probably also involved (Figure 4.1). Finally, based on the knowledge learned from this dissertation we will now be able reassure endemic populations and communicate to them that the AEs experienced after LF treatment are evidence that the medications that they have taken are working and killing harmful parasites in their bodies.
4.11 References


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70. Petit G, Diagne M, Marechal P, Owen D, Taylor D, and Bain O. Maturation of the filaria 
Litomosoides sigmodontis in BALB/c mice; comparative susceptibility of nine other 

71. Marechal P, Le Goff L, Petit G, Diagne M, Taylor DW, and Bain O. The fate of the 
filaria Litomosoides sigmodontis in susceptible and naturally resistant mice. *Parasite.* 

72. Morris CP, Evans H, Larsen SE, and Mitre E. A comprehensive, model-based review of 

73. Ash LR, and Riley JM. Development of subperiodic Brugia malayi in the jird, Meriones 

74. McNulty SN, Mitreva M, Weil GJ, and Fischer PU. Inter and intra-specific diversity of 

75. Beaver PC, Orihel TC, and Johnson MH. Dipetalonema viteae in the experimentally 
infected jird, Meriones unguiculatus. II. Microfilaremia in relation to worm burden. *J 
Parasitol.* 1974;60(2):310-5.

76. Michalski ML, Griffiths KG, Williams SA, Kaplan RM, and Moorhead AR. The NIH-

77. Bianco AE, and Denham DA. The action of diethylcarbamazine on the skin-dwelling 
microfilariae of Monanema globulosa (Nematoda: Filarioidea) in rodents. *Tropenmed 

78. Semnani RT, Keiser PB, Coulibaly YI, Keita F, Diallo AA, Traore D, Diallo DA, 
Doumbo OK, Traore SF, Kubofig J, et al. Filaria-induced monocyte dysfunction and its 

adverse reaction risks during mass treatment with ivermectin in loiasis-endemic areas. 

80. Mistry P, Laird MH, Schwarz RS, Greene S, Dyson T, Snyder GA, Xiao TS, Chauhan J, 
Fletcher S, Toshchakov VY, et al. Inhibition of TLR2 signaling by small molecule 
inhibitors targeting a pocket within the TLR2 TIR domain. *Proc Natl Acad Sci U S A.* 
2015;112(17):5455-60.

Leister W, Austin CP, and Xia M. Identification of known drugs that act as inhibitors of 
2010;79(9):1272-80.
**Figure 4.1:** Proposed signaling pathway of *Wolbachia* and filarial antigens.

[A] An updated overview of the proposed mechanisms by which *Wolbachia* contributes to the pathogenesis of adverse events after LF treatment. [B] An overview of the proposed mechanisms by which uncharacterized filarial antigens contribute to the pathogenesis of adverse events after LF treatment.

Figure is adapted from Taylor MJ, Cross HF, Ford L, Makunde WH, Prasad GB, and Bilo K. *Wolbachia* bacteria in filarial immunity and disease. *Parasite Immunol.* 2001;23(7):401-9.