MOLECULAR MECHANISMS OF LYMPHANGIECTASIA IN LYMPHATIC FILARIASIS

by

TIFFANY SUE WEINKOPFF

(Under the Direction of Patrick J. Lammie)

ABSTRACT

Lymphatic filariasis is caused by the parasitic nematodes *Wuchereria bancrofti*, and *Brugia malayi* which infect over 120 million people worldwide. Individuals harboring adult worms are typically asymptomatic, but patients exhibiting clinical manifestations of the disease like lymphedema are usually antigen-negative. Even though infected individuals appear asymptomatic, they exhibit subclinical manifestations such as lymphangiectasia or lymphatic vessel (LV) dilation. Lymphangiectasia is not restricted to the site of the worm nest but is seen along the length of the infected vessel suggesting a soluble product secreted by the worm could be responsible for the lymphatic pathology. Therefore, it is hypothesized that the worm excretory-secretory (ES) products are activating the lymphatic endothelial cells (LEC) lining the infected vessels. Initial studies characterized ES products and catalogued the ES protein constituents by mass spectrometry. Given the intimate interaction between the parasite and LECs, the ability of the ES products to directly activate LECs was analyzed, but there was a lack of evidence for the direct activation of LECs by ES products. Consequently, it was hypothesized that ES products indirectly activated LECs through an accessory cell type. Peripheral blood mononuclear cells (PBMC) from naïve volunteers were exposed to worm ES products. ES

products induced PBMCs from healthy volunteers to produce elevated levels of lymphangiogenic molecules such as IL-8, IL-6 and VEGF-A. CD14-positive monocytes were the primary producers of lymphangiogenic molecules in response to ES products. In addition, these lymphangiogenic mediators can induce LEC tubule formation *in vitro* and *in vivo*. Individuals from filarial-endemic regions of Haiti including individuals with active infection, lymphedema and endemic normal (EN) controls were also analyzed for the production of lymphangiogenic molecules in response to filarial ES products. All groups produced elevated levels of lymphangiogenic molecules in response to worm ES products and microfilaremic individuals exhibited a higher frequency of monocytes producing lymphangiogenic mediators compared to ENs. Taken together, these data suggest filarial ES products activate circulating cells to produce lymphangiogenic mediators that may contribute to the lymphangiectasia seen in infected individuals and thus the development of lymphedema.

INDEX WORDS: Lymphatic filariasis, Human, Parasite, Lymphangiectasia, Lymphedema, Lymphatic endothelial cells, Monocytes, Lymphangiogenesis

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DEDICATION

To my mom, Karen, who gave up her dreams so I could conquer the world…

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Background and Epidemiology of Lymphatic Filariasis

Lymphatic filariasis (LF) is caused by the filarial nematodes: *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. *W. bancrofti* is responsible for approximately 90% of infections worldwide while *B. malayi* is responsible for almost 10% of cases and *B. timori* can be found on several Indonesian islands (Michael et al., 1996). The parasite is transmitted by a mosquito; *W. bancrofti* adult worms live within lymphatic vessels (LV) of the human hosts whereas *B. malayi* adult worms live within the LVs of both human and non-human vertebrate hosts. In the LVs, adult worms release microfilariae; the microfilariae exit the LVs and travel to the peripheral blood to be taken up by a subsequent blood meal. The microfilariae mature into third-stage larvae (L3) which are transmitted to a vertebrate host or humans following a blood meal. The L3 develop into the adult worms which reside in the lymphatic vessels.

Filarial worms are a major cause of morbidity in many developing countries throughout Africa, Asia, the Americas and the Pacific (Michael and Bundy, 1997). It is estimated that 3 billion people are infected with helminthes and almost 250 million of these individuals are infected with one or more of filarial species (Ottesen, 1992). The filarial worms are also a significant impediment to socio-economic development in these regions. An estimated 120 million people in 73 countries are affected by LF with more than 1 billion people currently living in filariasis-endemic areas that are at risk for infection (Ottesen et al., 1997). Over 44 million individuals present with clinical pathology and an additional 76 million people carry parasites in

their blood and are affected by damage to their lymphatic systems (Ottesen et al., 1997). Clinical manifestations associated with the disease can include testicular hydrocoele, chyluria, acute episodes of adenolymphangitis, and lymphedema and elephantiasis of the extremities, breast, and male genitalia, but many of those who are currently infected with worms are asymptomatic (Dreyer et al., 2002).

Given the extreme burden of both infection and disease on the developing world, the World Health Assembly called on the member states of the World Health Organization (WHO) to eliminate LF as a public health problem in 1997 through resolution WHA50.29 (Molyneux and Taylor, 2001, Molyneux, 2003). This event led to the development of the Global Programme to Eliminate Lymphatic Filariasis (GPELF) which focuses on two main goals: 1) to use a single-dose annual mass drug treatment of an endemic population to interrupt transmission by reducing the reservoir of microfilariae and 2) to alleviate the suffering and disability of those suffering from clinical disease. The GPELF efforts are the largest infectious disease intervention based on mass drug administration (MDA) initiated to date (Weil and Ramzy, 2007). The effort to eliminate LF has been fueled by generous drug donations from GlaxoSmithKline and Merck and Company, Incorporated and the partnership between the public and private sectors including long-term commitments from the WHO and the World Bank as well as other governmental and non-governmental organizations (Molyneux and Taylor, 2001, Molyneux, 2003). During the first 8 years of MDA sponsored by the GPELF, over 1900 million antifilarial treatments in 48 countries have been given, preventing the spread of filarial infection to an estimated 6.6 million newborns and halting the progression to clinical morbidity in 9.5 million individuals who are already infected (Ottesen et al., 2008, Hooper et al., 2009, Molyneux, 2009).

Currently, there are three major chemotherapeutic agents used in the GPELF strategy to eliminate LF including diethylcarbamazine (DEC), ivermectin and albendazole. For most LF-endemic regions, albendazole is co-administered with DEC but in regions where LF is co-endemic with onchocerciasis (river blindness), albendazole is co-administered with ivermectin to prevent severe adverse reactions in patients infected with *Onchocerca volvulus* (Bradley et al., 2004, Rajkumar et al., 2005, Simonsen et al., 2008). DEC and ivermectin are thought to be mainly microfilaricidal with DEC possibly having partial macrofilaricidal effects. Unlike DEC and ivermectin, albendazole has no direct effect on microfilariae but it appears to suppress embryogenesis in the adult female worm (Bradley et al., 2004). Even though the mechanism of action of DEC remains elusive, work in non-filarial organisms has shown ivermectin acts on glutamate-gated chloride ion channels in invertebrate nerve and muscle cells causing parasite paralysis and death (Bradley et al., 2004, Geary, 2005). In addition, albendazole acts specifically on parasite tubulin inhibiting microtubule formation and thus mitosis, embryonation and egg hatching (Bradley et al., 2004). Given that these drugs mainly affect the microfilariae, they are used to prevent the transmission of LF rather than provide curative treatment by killing adult worms. As a result, new chemotherapies that act on the adult worm are needed to ensure global LF elimination. The potential for anti-*Wolbachia* agents like doxycycline is promising. Doxycycline depletes the worm's rickettsial endosymbiont, *Wolbachia*, leading to an almost complete absence of microfilariae after one year suggesting a long-term block in embryogenesis and/or macrofilaricidal activity, but currently this approach is limited by the three to six-week treatment regimen which is not optimal for field settings (Hoerauf et al., 2000, Bradley et al., 2004).

Currently LF is diagnosed by a variety of methods. Parasitologic methods rely on the collection of blood during the night for thick smear preparation and microfilarial detection by microscopy. This method is difficult and inconvenient, given the nocturnal periodicity of microfilariae in the peripheral blood and it is also somewhat insensitive. As a result, a rapid-format immunochromatographic test (ICT) for the detection of circulating antigen was developed which offers an attractive option since the antigen can be detected day and night. The ICT cards, pioneered by Weil et al., detect the presence of viable adult *W. bancrofti* and are much more sensitive than conventional methods of microfilaria detection alone (Freedman, 1998). There is also an antigen test based on the monoclonal antibody Og4C3 that is used in laboratories, but the sandwich ELISA format is quite labor-intensive and requires laboratory facilities (Weil et al., 1997). Antibody detection by ELISA against a variety of antigens including Bm14 is commonly used in the laboratory (Chandrashekar et al., 1994). However, antibody detection assays can cross-react with other species and current infection can not be differentiated from past infections (Lammie et al., 2004). To date, the most effective methods to measure transmission rely on entomologic surveys using PCR to determine infection rates in potential vectors, but this approach is unrealistically expensive for many programs and requires specialized laboratory capacity. Together, all of these assays help to provide prevalence information on those individuals who are currently infected, but elimination programs need an assay that can act as a marker of transmission for both asymptomatic microfilaremic persons as well as those with disease. Assessing transmission by evaluating exposure to LF in children is possible in principle, but assays with the requisite sensitivity and specificity have not been appropriately validated.

This ideal antigen could be used to measure the success of each program in a globally standardized approach in school-age children to provide evidence that transmission has been interrupted.

1.2 Filarial Disease: Lymphangiectasia and Lymphedema

The lymphatic system consists of a series of vessels that are lined by endothelial cells (EC) which transport fluids. The lymphatics serve several functions; their primary function is to drain excess interstitial fluids to prevent tissue swelling. The blood flows through capillaries and into adjacent tissues and then exits through veins; however, about 10% of the fluid filtered by the capillaries gets caught in the tissues. About 3 liters of fluid per day remain in the tissues and without proper drainage and transport to the circulatory system by the lymphatic system, this fluid accumulation could be life-threatening (Tortora and Grabowski, 2003). The fluid that accumulates is also rich in proteins, so it serves as a substrate for pathogens that penetrate the skin barrier. Besides tissue homeostasis, the lymphatics also play a role in fat absorption in the digestive system and immune surveillance.

LF is an infection with varying degrees of clinical lymphatic pathology. Individuals can exhibit overt clinical symptoms such as lymphedema and hydrocele or be asymptomatic but exhibit microfilaremia. In human filarial infections, living adult worms induce alterations in host tissues with subclinical lymphangiectasia being the most common. Lymphangiectasia can be defined as a dilation of the LV that can result in a modification in lymphatic drainage with varying severity. Asymptomatic microfilaremic individuals do not appear to demonstrate any obvious clinical manifestations, but they do present with hidden subclinical complications such as microscopic haematuria and/or proteinuria, dilated and tortuous lymphatics and scrotal lymphangiectasia in men (Dreyer 1992, Freedman et al., 1994, 1995, Noroes et al., 1996a,

1996b, Nutman et al., 2001). For instance, ultrasonographic examination of the scrotal region of 14 asymptomatic Brazilians revealed that 50% of the microfilaremic individuals showed lymphatic dilation and tortuosity (Amaral et al., 1994). It is thought that the early phase of lymphatic pathology is characterized by lymphangiectasia and only with the addition of secondary co-factors will the lymphatic dilation develop into an overt chronic lymphedema. Dreyer et al. believe that such factors may include permissive factors such as heavy worm burdens or non-permissive factors such as host response, DEC treatment and bacterial infections (Figueredo-Silva et al., 2002).

Lymphangiectasia can be detected by radionuclide lymphoscintigraphy, magnetic resonance imaging, ultrasonography and histopathologic methods (Case et al., 1992). It is thought that all infected individuals will eventually develop a localized lymphangiectasia given sufficient time (Dreyer et al., 2002). Even though most microfilaremic individuals do not present with any overt clinical pathology, abnormal lymphatics are present in 69% of limbs of infected persons by static lymphoscintigraphy and in 100% of limbs by dynamic flow lymphoscintigraphy, which are sensitive indicators of lymphatic dysfunction (Freedman et al., 1994, 1995; Dissanayake et al., 1995). In addition, studies on superficial skin punch biopsies have revealed that 78% and 68% of limbs from patients with clinical pathology and asymptomatic microfilaremia, respectively, contained lymphatic vessels that were abnormally dilated as detected by antibody staining (Freedman et al., 1995, 1998). Even infected children as young as 3 years old presented with dilated lymphatics as measured by lymphoscintigraphy suggesting that subclinical pathology can occur at a very early age (Shenoy et al., 2007, 2008).

In LF, the LVs become dilated and flow is impaired, leading to improper drainage of interstitial fluids (Vaqas and Ryan, 2003). Lymphedema develops from mild lymphangiectasia

as a result of lymph fluid accumulation in the tissues following damage to LVs (Ottesen, 1992). The progression of lymphedema to the more chronic elephantiasis gradually occurs over many years from mild lymphatic dilation to complete dysfunction. The causes for the development of the clinical manifestations associated with the disease are unclear; however, many hypotheses have been propounded. Many have proposed parasitic factors as the cause for disease. More specifically, mechanical damage or excretory-secretory (ES) products secreted by the worm may have an influence on the surrounding lymphatic tissues or the host's immune response (Ottesen, 1992; WHO 1992). Dreyer et al. have proposed that the lymphangiectasia caused by the living adult worm may be a major risk factor for the development of disease due to the lymphatic dysfunction attributed to the worm (Dreyer et al., 2000a, 2000b). The progression of chronic lymphedema has also been attributed to secondary bacterial and fungal infections in animal models and humans (Bosworth and Ewert 1975, Barbee et al., 1977, Ottesen, 1994, Olszewski et al., 1997, Dreyer et al., 2000a, 2000b).

The immune status of an individual or host immune responses have also been implicated in the development of the various clinical manifestations associated with LF (Ottesen, 1992). Since antifilarial humoral and cell-mediated immune responses, characterized by high levels of antifilarial IgG1, IgG2, IgG3 and IFNγ, are elevated in patients with lymphedema, it is thought that antifilarial immunity can be associated with the development of disease; and that parasite antigens stimulate T cells and thus an inflammatory response which promotes lymphatic dysfunction and contributes to pathogenesis (Lammie et al., 2002).

Host genetics have also been implicated in the development of lymphedema. Almost all individuals with active infection are asymptomatic and exhibit lymphangiectasia, but it is not known why only a small proportion of exposed persons display chronic pathology (Lammie et

al., 2002). As a result, it is hypothesized that some hosts are genetically predisposed to disease progression and studies on Haitian pedigrees suggested that lymphedema of the leg aggregates within families and antifilarial antibody responses cluster in high risk families (Cuenco et al., 2001, Yang unpublished).

The progression of lymphedema to elephantiasis has also been suggested to be a result of hypersensitive immunopathology to dead worms and the antigenic material released with their death (von Lichtenberg, 1957, Warren, 1971). Adult worms may die as a result of natural attrition or DEC treatment. Furthermore, it has been shown that degenerating or dead adult worms induce an inflammatory response in the LV resulting in the formation of granulomatous nodules or episodes of acute filarial lymphangitis (Jungmann et al., 1991, Dreyer et al., 2000b). It has been proposed that this inflammatory response to the degenerating worms could be due to the release of the *Wolbachia* endosymbiont; however, worm death alone is likely not sufficient to induce chronic pathology (Taylor et al., 2000, 2001). Even though worm death initiates an inflammatory response and thus an infiltrate of immune cells that transiently occlude the LVs, collateral lymphatics may compensate for the acute loss of function (Dreyer et al., 2000b). Eventually recanalization is thought to restore some lymphatic flow around vessels containing lesions and the granuloma seems to decrease in size with time allowing the lymphatic lumen to re-open (Dreyer et al., 2000b, Figueredo-Silva et al., 2002).

Experimental animal models have both supported and refuted the role of the host in the development of disease. Congenitally athymic nude mice (C3H/HeN) are highly susceptible to infection with *B. pahangi* while wild type mice show a strong thymus-dependent resistance with termination of larval infection (Vincent et al., 1984). Progressive lymphangiectasia was seen in nude and SCID mice compared with an arrest in lymphangiectasia in wild type mice suggesting

lymphatic pathology is not a result of the immune response, but is rather a consequence of the presence of the parasite and its products (Vincent et al., 1984, Nelson et al., 1991). This dilation is reversed in nude mice by removing or killing the adult worms (Vickery et al., 1983, 1991). Additional studies using a modified SCID mouse model indicate that a functional immune system is not necessary for lymphatic dilation, retention of lymph and inflammatory changes including lymphangitis and lymphadenitis (Vincent et al., 1984). In clinical studies, Shenoy et al. demonstrated that lymphatic dilation has already occurred in filarial-infected children as young as 3 years of age and that worm death by DEC treatment can reduce the subclinical lymphatic pathology (2008, 2009). Importantly, lymphangiectasia is not strictly limited to the exact location of the worm nest but can be found all throughout an infected vessel suggesting there could be parasite-derived or induced soluble factors that can be transported the length of the vessel to mediate the effects (Amaral et al., 1994).

Others argue that the development of lymphangiectasia is mediated by the immune response. Vickery et al. showed that reconstituting *B. malayi*-infected nude mice by the adoptive transfer of primed spleen cells from chronically infected mice could induce worm killing and the development of lymphatic lesions, suggesting the reconstitution of an immune system could restore lymphatic pathology not seen in nude mice alone (Vickery et al., 1991). This result argues that the immune system is responsible for the lymphatic pathology that follows worm death.

Several previous studies have attempted to evaluate the role of *B. malayi* and ES products on ECs. In infected patients, vessels containing live adult worms are distended and devoid of any inflammatory response, however there are some lymphocytes present as normal components of lymph (Figueredo-Silva et al., 2002). The walls of infected vessels appear to have regions of

fibrosis that alternate with areas of limited smooth muscle cells while other areas display muscle cell hyperplasia (Figueredo-Silva et al., 2002). Furthermore, ECs appear preserved but electron microscopy reveals bulging nuclei, an increased number of pinocytotic vesicles and an abundance of collagen fibers (Figueredo-Silva et al., 2002). In contrast, transmission electron microscopy of lymphatic endothelial cells (LECs) from *B. malayi*-infected cats shows a decrease in the number of cytoplasmic vesicles compared to uninfected controls, however irregularly large vesicles were commonly seen in infected vessels (Sakamoto et al., 1985). A parallel study investigating LECs from *B. pahangi*-infected jirds found that there was a decrease in the proportion of cytoplasm occupied by vesicles and in the number of cytoplasmic vesicles in LECs from infected vessels compared to uninfected vessels (Sakamoto et al., 1988). However, the study also reported an increase in the area of vacuoles in LECs from infected vessels (Sakamoto et al., 1988). These changes in EC vesicles and vacuoles may influence the ability of LECs to drain and transport protein-rich fluids through the lymphatic system (Sakamoto et al., 1985, 1988).

In animal models, it has been shown that lymphatics of parasitized nude mice become highly tortuous with an accumulation of highly proteinaceous lymph fluid and the limbs exhibit an elephantoid appearance (Vickery et al., 1985). Histological examination of these dilated lymphatics from infected nude mice showed a cuboidal endothelial lining with small nonobstructive lymph thrombi composed of small mononuclear cells and multinucleated giant cells within the lumen (Vickery et al., 1985). The endothelium of the dilated vessels was perturbed, scalloped, bulbous and highly indented (Rao et al., 1996). Furthermore, the removal of worms reversed the process suggesting that viable *B. malayi* exert direct pathological effects upon the lymphatics (Sakamoto et al., 1985, Vickery et al., 1985). Similar tissue alterations were

seen in dilated LVs of cats infected with *B. malayi* (Fader et al., 1984). Vascular abnormalities have also been evaluated in the ferret experimental model for LF. Microscopy of dilated lymphatics of *B. malayi*-infected ferrets showed that dilation was greatest near the worm nests with endothelial ballooning, swelling, scarring, thickened walls and valves and thrombus formation which also supports the proposal that the presence of living adult worms and their ES products alters LV tissue in a gradient-dependent manner (Case et al., 1991). Taken together, these observations suggest that LECs maybe prime targets of parasite-derived factors which induce the development of clinical pathology (Rao et al., 1996).

Previous studies have also shown that filarial products not only alter the morphological characteristics of LECs, but also physiological functions of ECs. Filarial parasites are capable of modulating vascular activity by inducing changes in rat abdominal aorta EC behavior; both endothelial-dependent relaxation and the frequency of spontaneous contractions were disrupted (Kaiser et al., 1991, 1996). Given the lack of a central pump throughout the lymphatic system, spontaneous contractibility most likely serves a critical function in the peristalsis necessary for lymphatic propulsion; moreover, this depressed contractibility seen in co-cultures containing ECs and parasites could correlate with the altered lymphatic function seen in lymphedema patients (Kaiser et al., 1996). Overall, parasite-derived factors have been shown to alter the physiologic functions of ECs *in vitro*.

Some authors have proposed that parasites induce endothelial and connective tissue proliferation *in vivo* which in turn causes the thickening of the endothelium (von Lichtenberg, 1987, Buck, 1991). It is thought that the hyperplastic nature of ECs and the low opening pressure of dilated LVs indicate abnormal multiplication of LECs (Rao et al., 1996). The cuboidal ECs seen in *B. malayi*-infected nude mice suggest an increase in the multiplication of

LECs may also contribute to lymphangiectasia (Vickery et al., 1985). However, since this phenomenon could not be reproduced *in vitro* with human umbilical vein endothelial cells (HUVEC), the authors suggested the lack of proliferation could be due to the ES products suppressing the activity of growth factors or the parasite might have inactivated or absorbed the growth factors from the medium (Rao et al., 1996). It is interesting to note that the addition of lymph, but not serum, from dilated lymphatics of infected mice was able to increase the rates of cellular division by ECs seen *in vitro* (Rao et al., 1996).

1.3 Pathogenesis of Lymphatic Filariasis

Filarial worms have been noted for their longevity in human infections with an average lifespan of around six years. It is generally accepted that the worms facilitate their survival by modulating the host response to their advantage (Harnett and Harnett, 2006a, 2006b). This has been attributed to a defective lymphocyte proliferative response, referred to as lymphocyte hyporesponsiveness. This lack of an appropriate immune response was first reported in the 1970s when lymphocytes isolated from infected individuals failed to respond to filarial antigens and also again in animal models (Ottesen et al., 1977, Weiss, 1978). In general the immune dysfunction seen in microfilaremic patients has been characterized by low serum levels of parasite-specific antibodies and impaired antigen-specific T- and B-cell responses to parasite antigens *in vitro* (Ottesen et al., 1977, Piessens et al., 1980, Hussain et al., 1981, Lammie and Katz, 1983, Nutman et al., 1987, Nielsen et al., 2002a). The roles of various immune effectors have been investigated to define their contribution to the hyporesponsiveness seen in filarial infections, but the direct involvement of LECs has not been identified. Given their direct contact with the worms and their location, LECs may be a prime target of parasite products.

The cause of the lymphocyte hyporesponsiveness seen in filarial infections is not known; however, it is likely that all stages of the parasite are capable of suppressing the immune response (Harnett and Harnett, 2006b). Studies using hamsters infected with *Acanthocheilonema viteae* and jirds infected *B. pahangi* demonstrated that microfilaremia was associated with impaired lymphocytic proliferation to filarial antigens and B- and T-cell mitogens (Weiss, 1978, Weller et al., 1978, Lammie and Katz, 1983, Harnett and Harnett, 2006b). In humans, the inhibition of an antigen-specific lymphocyte response was associated with microfilaremia and the elimination of microfilariae by chemotherapeutic treatments was linked with the recovery of lymphocyte proliferative responses (Ottesen et al., 1977, Piessens et al., 1981, Mahanty and Nutman, 1995). However, more recent studies in humans have shown that the lack of an immune response correlates better with levels of circulating antigen than with microfilaremia (Dimock et al., 1996, King, 2001). Besides microfilariae, ES products have also been shown to induce lymphocyte hyporesponsiveness with *in vitro* worm culture products being capable to inhibit lymphocyte proliferation (Wadee et al., 1987, Elkhalifa et al., 1991, Hartmann et al., 1997).

The mechanisms responsible for the immune dysregulation are not fully understood. Proposed mechanisms have included a decreased number of lymphocytes, the presence of tolerized lymphocytes and a decrease in co-stimulatory signals required for immunologic activation. A decrease in parasite-specific lymphocyte precursor frequency was measured in individuals with microfilaremia and asymptomatic microfilaremic individuals had significantly lower numbers of IFNγ-secreting cells, produced less IFNγ mRNA and secreted less IFNγ than persons with clinical pathology (King et al., 1992, King et al., 1993, Maizels et al., 1995, Mahanty et al., 1996, 1997, Dimock et al., 1996, Ravichandran et al., 1997, Nielsen et al.,

2002a). In addition, lymphocyte apoptosis was seen in *B. pahangi*-infected mice (O'Connor et al., 2003). Immune suppression could also be the result of clonal deletion that had occurred from *in utero* exposure since children born to microfilaria-positive mothers continued to be hyporesponsive to filarial antigens throughout their lives (Weil et al., 1983, Lammie et al., 1991, Steel et al., 1994).

The lack of lymphocytes may not be the only factor involved in the diminished immune response, but the lymphocytes may be present, yet dysfunctional. For instance, there is an increased surface expression of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) in patients with microfilariae (Steel and Nutman, 2003). CTLA-4 is a receptor for the co-stimulatory molecule B7 on antigen presenting cells (APCs) and it delivers an inhibitory signal to T cells, in contrast to its homologue CD28 which activates the T cell. B lymphocytes, along with monocytes, from filarial-infected patients displayed decreased activation and cytokine production upon TLR stimulation implying a state of immunologic unresponsiveness (Babu et al., 2005b).

Antibody polarization may also contribute to the immune modulation seen in filarial infections. In general, patients with clinical pathology exhibit higher levels of specific IgG1, IgG2, IgG3 and IgE compared to asymptomatic microfilaremic individuals; and asymptomatic microfilaremic persons display higher levels of IgG4 serum antibodies (Kurniawan et al., 1993, Yazdanbakhsh et al., 1995, Zhang et al., 1999, Nielsen et al., 2002b). Higher IgG4:IgE ratios are thought to characterize those with active infection and parasite protection where antibodies characterized by the opposite ratio may play a role in parasite killing (Nielsen et al., 2002b). In addition, lower levels of IFNγ were found in groups with high IgG4:IgE or in individuals with active infection (Nielsen et al., 2002b).

Inhibitory effectors such as T-regulatory cells (Tregs) may play a role in the lymphocyte hyporesponsiveness seen in LF (Taylor et al., 2005). Taylor et al. showed that the susceptibility seen in the BALB/c mouse strain, but not the C57B/6 strain, correlates with the increased expression of Foxp3 (2005). Furthermore, the targeting of CD4+ T cells with antibodies against CD25 and GITR found on Tregs provides an immunological cure for filarial infection in the BALB/c model (Taylor et al., 2005). In addition, molecules released by such regulatory cell populations such as IL-10 and TGF-β can be neutralized with antibodies to restore some of the diminished T cell proliferation (King et al., 1993, Mahanty et al., 1995, 1996, 1997). Therefore, lymphocyte proliferation can be inhibited by the induction of a regulatory T cell population.

It is hypothesized that the worm-induced production of anti-inflammatory cytokines like IL-10 may help to prevent an excessive Th2 response (van den Biggelaar et al., 2000, Gillan and Devaney, 2005). Asymptomatic microfilaremic patients as well as those with clinical pathology have similar levels of IL-4 and IL-5, typically Th2 cytokines (King et al., 1993, Maizels et al., 1995, de Almeida et al., 1996, Dimock et al., 1996, Ravichandran et al., 1997). Taken together, the increased antigen-specific IL-4:IFNγ ratio seen in asymptomatic microfilaremic individuals may indicate a lack of Th1 as opposed to a predominant Th2 response since the Th2 cytokines are not changed between groups (Freedman, 1998).

The activity of APC (Allen et al., 1996, Osborne and Devaney, 1999) has been shown to be critical for immunosuppression. *In vitro* T cell proliferation to a non-filarial antigen, conalbumin, was inhibited in the presence of peritoneal exudate cells from *Brugia*-infected mice (Allen et al., 1996). Human monocytes from microfilaremic individuals produce large amounts of IL-10 spontaneously *ex vivo* and are unable to respond to LPS (Mahanty et al., 1996, Sasisekhar et al., 2005). Chronic exposure to filarial antigens is thought to immunologically

deactivate monocytes/macrophages as assessed by their inability to kill *Toxoplasma* and produce TNFα (Nasarre et al., 1998). Taken together, these findings suggest that APCs from microfilaremic persons will not function optimally in presenting antigen to CD4+ T cells (Nutman et al., 2001). This hypothesis is supported by evidence of decreased expression levels of CD80 and CD86 on monocytes from microfilaremic patients compared to uninfected persons (Nutman et al., 2001). CD80, also called B7-1, and CD86, called B7-2, are co-stimulatory molecules expressed by APCs that bind the CD28 receptor on T cells. The receptor-ligand binding of such co-stimulatory molecules is required for T cell activation and the absence of this secondary signal may lead to T cell anergy and thus the dysfunctional immune response seen in filarial infections.

The ability of immunological effectors to migrate into the site of infection can also determine the extent of an immune response. Studies have shown that immune cells from asymptomatic microfilaremic patients are less likely to reach the site of infection compared to those with clinical pathology. Babu et al. showed decreased levels of chemokines in the serum combined with decreased levels of the chemokine receptor CCR9 on T and B cells from microfilaremic individuals which supports a role for these molecules in the hyporeactive immune response (Babu et al., 2005a). In addition to reduced production of chemotactic factors, the ability to migrate is also affected. Lymphocytes from infected individuals were more likely to be dysfunctional in their migration to the site of infection and thus, unable to initiate an appropriate immune response (Freedman et al., 1996, Plier et al., 1997). Research on the role of the ECs and chemokines at infection sites should provide greater knowledge and understanding in the dysfunctional immune response.

It has also been shown that filarial ES products can directly inhibit lymphocyte proliferation; a phosphorylcholine-containing glycoprotein discovered in the rodent filarial nematode *A. viteae*, called ES-62, inhibits polyclonal activation of murine B cells and has also been shown to inhibit the ability of macrophages to produce Th1 cytokines like IL-12 and TNF-α (Weil and Liftis, 1987, Harnett et al., 1989, 1993, 1999, Goodridge et al., 2001, Stepek et al., 2004). ES-62 can regulate dendritic cell (DC) maturation to elicit Th2-polarized responses and induce spleen and B1 B cells to produce IL-10 (Whelan et al., 2000, McInnes et al., 2003, Wilson et al., 2003). In addition, ES-62 induces the production of the Th2-associated IgG1 isotype but not the IgG2a isotype typically associated with a Th1 response in mouse models (Harnett et al., 1999). Since ES-62 is found in the L4 and adult stages, this supports the hypothesis that most stages of the parasite are capable of suppressing the immune response, rather than just microfilariae (Harnett et al., 1989).

ES-62 was also found to interfere with cell signaling resulting in the decreased production of pro-inflammatory cytokines IL-6, IL-12 and TNFα by inhibiting p38 MAPK activity; ES-62 also initiated ERK MAPK signaling which transduces negative feedback inhibition of IL-12p40 production (Goodridge et al., 2003). It has also been shown that ES-62 interferes with lymphocyte function by modulating PKC signaling and disrupting normal signaling cascades and nuclear transcription patterns which in turn suppresses B cell proliferation following antigen receptor ligation (Deehan et al., 1997, 1998, 2001, Harnett and Harnett, 2006a). Similar results were observed using ES-62 on the human Jurkat T cell line (Harnett et al., 1998).

Other ES products have been shown to display similar inhibitory characteristics. The *A. viteae* ES antigen Av17 is a cysteine protease inhibitor that suppresses T cell proliferation in the

mouse model (Hartmann et al., 1997). The filarial nematode *O. volvulus* also secretes a cysteine protease inhibitor called onchocystatin or Ov17 that suppresses immune cell proliferation (Schonemeyer et al., 2001). In addition, filarial molecules that mimic host factors have also been shown to possess suppressive activity including homologues of TGF-β and the macrophage migration inhibitory factor (MIF) (Gomez-Escobar et al., 1998, Pastrana et al., 1998). Mammalian MIFs are typically associated with pro-inflammatory responses, and filarial MIFs have similar properties; the *Brugia* MIF homologue was shown to induce monocyte/macrophage and eosinophil recruitment (Pastrana et al., 1998, Falcone et al., 2001). However, it does not seem consistent for the parasite to induce an inflammatory response with the secretion of MIF homologues in the context of a parallel induction of immunological tolerance for long-term survival (Zang et al., 2002). Overall, these filarial modulatory molecules mitigate the host response and contribute to worm persistence by modifying the host cytokine network (Zang et al., 2002).

In conclusion, there are probably several mechanisms including both parasite and host-mediated processes that are regulating the pathogenesis of filarial infection (Harnett and Harnett, 2006b). Regardless of the mechanisms, the role of the LEC has not been evaluated. Given their proximity to adult worms, LECs could be prime targets for parasite ES products. Stimulation of the LECs by these products may alter their LEC function contributing to the development of lymphangiectasia and thus the development onto filarial lymphedema.

1.4 Molecular Mechanisms of Lymphangiectasia and Lymphangiogenesis

The lymphatic system has remained secondary to the blood vascular system as a research topic, but now that the lymphatics have been shown to play crucial roles in cancer, wound healing and inflammation, the field is quickly emerging. Although the blood and lymphatic

vessels operate in parallel, share some anatomical features and show comparable transcriptome levels by microarray analyses, they also differ in many ways (Petrova et al., 2002, Hirakawa et al., 2003). The blood vascular system is a closed network where the lymphatics are an open-ended linear system from which fluid is drained from the interstitial space of peripheral tissues to be transported from thin capillaries into larger collecting lymphatics. From here, fluid is emptied into the right lymphatic duct and the thoracic duct before draining into the blood circulation via the right and left subclavian veins. In contrast to blood capillaries, the lymphatic capillaries are thin-walled terminal end tubes that lack smooth muscle cells/pericytes and a continuous basement membrane. But the LVs do contain large interendothelial valve-like openings and anchoring filaments that directly connect the vessels to the extracellular matrix (ECM) and maintain luminal shape. The lymphatics tend to have a wider and more irregularly-shaped lumen lined by a single layer of ECs that are thought to remain collapsed under physiologic conditions until interstitial pressure increases due to fluid drainage. As a result, the anchoring filaments exert tension on the LECs to pull open the overlapping cell junctions so that fluids are able to enter the lymphatic lumen. Once the interstitial fluid enters the LV lumen, the pressure difference across the wall decreases and the vessel closes. (Pograbinska et al., 2002, Lohela et al., 2003, Pepper et al., 2003, Hong et al., 2004a, Saharinen et al., 2004)

Given their biological function, LVs exhibit greater permeability so that they are more effective at removing protein-rich fluids from intracellular spaces. Compared to blood endothelial cells (BEC), LECs also contain a greater number of cytoplasmic vesicles and express genes for protein metabolism, sorting and trafficking indicating a robust role in molecule transport (Leak, 1972, 1976, Pograbinska et al., 2002). Unlike the blood vasculature, there is no central pump. LVs rely on respiratory movements, adjacent skeletal muscles and the

contractility of the smooth muscle cells surrounding the larger collecting LVs to propel lymph (Muthuchamy and Zawieja, 2008). When a LV becomes stretched with fluid, the wall of the vessel automatically contracts and valves maintain a uni-directional flow throughout the network. (Pograbinska et al., 2002, Lohela et al., 2003, Pepper et al., 2003, Hong et al., 2004a, Saharinen et al., 2004)

Much of the previous work on ECs focused on BECs, but only recently the field of LEC biology has taken shape. The identification and increased availability of lymphatic-specific EC markers such as LYVE-1, Prox-1, podoplanin and vascular endothelial growth factor receptor-3 (VEGFR-3) have completely transformed the field within the past ten years. In addition, the discovery of the vascular endothelial growth factors (VEGF), a family of growth factors that regulate vascular EC proliferation, angiogenesis, lymphangiogenesis, vasculogenesis and vascular permeability, has also revolutionized the field (Adams and Alitalo, 2007). Overall the VEGF family contains six known members that are highly related structurally including placental growth factor (PIGF), VEGF-A, VEGF-B, VEGF-C, VEGF-D and viral VEGF homologues known as VEGF-E. The disruption of genes encoding VEGF or any of the three receptors of the VEGF family (VEGFR-1, VEGFR-2 and VEGFR-3) results in embryonic lethality due to insufficient blood vessel development (Fong et al., 1995, Shalaby et al., 1995, Dumont et al., 1998, Eriksson and Alitalo, 1999, Makinen et al., 2001a, Shibuya, 2001).

Lymphatic-specific markers include LYVE-1, a lymphatic endothelial transmembrane receptor for the ECM/lymphatic fluid glycosaminoglycan hyaluronan (HA) (Banerji et al., 1999, Nisato et al., 2004). HA is a large mucopolysaccaccharide polymer that comprises the ECM and undergoes constant turnover (Jackson et al., 2001, 2003, Hong et al., 2004a). HA is transported through the lymph to the lymph nodes where it is partially degraded before it reaches the liver

for complete digestion (Hong et al., 2004a). HA turnover increases during tissue injury and the by-products induce an inflammatory response, leading to angiogenesis, chemokine production and DC recruitment (Hong et al., 2004a). Even though LYVE-1 is expressed on all embryonic LECs and later restricted to lymphatic capillaries in the adult, it is not essential for lymphatic growth as LVYE-1 deficient mice are viable with normal lymphatics (Jackson, 2004, Makinen et al., 2005, Adams and Alitalo, 2007).

Another lymphatic-specific marker is podoplanin, a glomerular podocyte transmembrane mucoprotein that is expressed by LECs, but not BECs, *in vivo* and *in vitro* (Wetterwald et al., 1996, Kriehuber et al., 2001, Petrova et al., 2002, Hirakawa et al., 2003, Schacht et al., 2003, Hong et al., 2004a, Nisato et al., 2004). Mice deficient in podoplanin die at birth due to lung failure; these mice exhibit malformed LVs and diminished tissue drainage (Ramirez et al., 2003, Schacht et al., 2003, Adams and Alitalo, 2007). According to overexpression studies and siRNA knockdowns, podoplanin seems to play a role in LEC migration, adhesion and tube formation (Schacht et al., 2003).

Prox-1, a homeodomain transcription factor regulating early lymphatic development, can also be used to identify LECs (Nisato et al., 2004, Adams and Alitalo, 2007). Overall, it is thought that Prox-1 might be involved in specifying lymphatic cell fate (Wigle et al., 2002, Hong et al., 2004a). This transcription factor is the first known regulator of lymphangiogenic growth in the mouse embryo and Prox-1 knockout mice die around embryonic day 15 lacking LVs (Wigle and Oliver, 1999, Wigle et al., 2002, Adams and Alitalo, 2007). In addition, the ectopic expression of Prox-1 has been shown to be sufficient to reprogram transcriptome of BECs to adopt a lymphatic phenotype by repressing BEC-specific genes and inducing lymphatic-specific genes (Hong et al., 2002, Petrova et al., 2002).

VEGFR-3, also known as Flt4, is a transmembrane tyrosine kinase receptor typically restricted to the lymphatic endothelium in adults, but has also been shown to be expressed in blood vasculature during embryogenesis, and in wound healing and tumors (Kaipainen et al., 1995, Partanen et al., 1999). Embryos deficient in VEGFR-3 die due to defective blood vessel development leading to cardiovascular failure suggesting that VEGFR-3 plays a crucial role in the development of the cardiovascular system prior to the emergence of LVs (Dumont et al., 1998). Postnatally, VEGFR-3 occurs mainly in the lymphatic endothelium where it binds VEGF-C and VEGF-D and promotes the growth, survival and migration of LECs (Makinen et al., 2001a). The blocking of VEGFR-3 signaling with a soluble VEGFR-3 protein caused the regression of developing lymphatics by inducing EC apoptosis (Makinen et al., 2001b). Given that mutations in *Vegfr-3* are embryonically lethal in mice, it has been difficult to study the molecule's role in lymphatic development, but skin-specific overexpression of VEGFR-3 in transgenic mice is sufficient to promote lymphangiogenesis or the development of new LVs (Veikkola et al., 2001). It should also be noted that mutations in the human gene *Vegfr-3* are responsible for hereditary lymphedema type I (Alitalo et al., 2005, Adams and Alitalo, 2007).

The ligands of VEGFR-3, VEGF-C and VEGF-D, selectively promote the growth, survival and tubule formation of LECs (Joukov et al., 1996, Lee et al., 1996, Achen et al., 1998, Makinen et al., 2001a, Podgrabinska et al., 2002). Therefore, these molecules have been found to be potent regulators of lymphangiogenesis (Joukov et al., 1996, Lee et al., 1996, Jeltsch et al., 1997, Achen et al., 1998). However, further proteolytic processing of VEGF-C can produce isoforms capable of binding VEGFR-2 so that this factor can also play a role in angiogenesis and increase vascular permeability (Joukov et al., 1996, 1997). VEGF-C shares 30% identity with VEGF-A and VEGF-D is structurally 48% identical to VEGF-C (Joukov et al., 1996,

Schoppman, 2005). *Vegf-c*-mutant embryos die from fluid accumulation in tissues due to a lack of lymphatic vasculature and mice containing a single *Vegf-c* allele exhibit lymphatic hypoplasia and lymphedema (Karkkainen et al., 2004). Given the requirement for VEGF-C to promote the budding and proliferation of Prox-1-expressing LEC from the cardinal vein, *Vegf-c* mutant ECs commit to the lymphatic lineage but they do not sprout to form LVs (Karkkainen et al., 2004). The deletion of *Vegf-d* does not affect the development of the lymphatic vasculature but recombinant VEGF-C and VEGF-D can rescue the impaired vessel sprouting in *Vegf-c* deficient embryos (Karkkainen et al., 2004, Baldwin et al., 2005, Alitalo et al., 2005).

In a variety of experimental systems, VEGF-C and VEGF-D have been shown to induce lymphangiogenesis and angiogenesis. In a chick chorioallantoic membrane model, VEGF-C induced LEC proliferation and the development of new lymphatics (Oh et al., 1997). Over-expression of VEGF-C in the skin of transgenic mice stimulated LEC proliferation and hyperplastic LVs; in addition, subcutaneous adenovirus delivery of VEGF-C to mice induced lymphangiogenesis (Jeltsch et al., 1997, Enholm et al., 2001). In a mouse cornea model, VEGF-C induced LV growth and these effects could be inhibited by the administration of antibodies against VEGFR-3 (Kubo et al., 2002). Using a more selective approach, transgenic mice overexpressing a VEGFR-3-specific VEGF-C induced the growth of LVs in the skin (Veikkola et al., 2001). Over-expressing VEGF-D induces lymphangiogenesis in the skin of transgenic mice (Veikkola et al., 2001). Furthermore, adenovirus delivery of VEGF-D protein induced EC proliferation and lymphangiogenesis in rat skin and in rabbit hindlimb muscle and adenovirus-encoded VEGF-D served as the most potent member of the VEGF family (Byzova et al., 2002, Rissanen et al., 2003).

Likewise, in a mouse tumor model human VEGF-D induced angiogenesis, lymphangiogenesis, and metastatic spread through the lymphatics (Stacker et al., 2001).

Interestingly, VEGF-C expression has been identified in about half the human cancers analyzed and the levels of VEGF-C expression have been correlated with lymph node (LN) metastasis in multiple cancers such as thyroid, gastric, colorectal and lung (Salven et al., 1998, Schoppman, 2005). Tumor cells express VEGF-C and have been shown to play a role in tumor lymphangiogenesis and metastasis (Mandriota et al., 2001, Skobe et al., 2001a, 2001b). In addition, enlarged LVs as well as lymphangiogenesis have been reported in peritumoral areas and the number of tumor-associated lymphatics correlated with LN metastases (Stacker et al., 2002, Pepper et al., 2003). The administration of tumor cells expressing VEGF-C promoted the growth of tumor-associated lymphatics and these effects could be inhibited by a soluble VEGFR-3 fusion protein (Karpanen et al., 2001). Taken together, these data suggest that recombinant VEGF-C protein or tumor cells can initiate LV growth and that these effects are mediated primarily through VEGFR-3 signaling.

VEGF-C and VEGF-D act as mediators of lymphangiogenesis which may increase the propensity for cancer to spread via the lymphatic system (Stacker et al., 2002, Pepper et al., 2003). It is thought that tumor cells are capable of migrating through the lymphatics, as opposed to the blood vasculature, because lymphatics have looser junctions and a discontinuous basement membrane (Alitalo and Carmeliet, 2002). As a result, VEGF-C offers an attractive target for the inhibition of tumor lymphangiogenesis and LN dissemination via a soluble VEGFR-3 fusion protein or neutralizing antibodies (Karpanen et al., 2001, He et al., 2005, Schoppman, 2005).

The role of VEGF-A in lymphangiogenesis is not as well characterized as VEGF-C or VEGF-D. VEGF-A signaling through VEGFR-2 is considered to be the main receptor that

mediates BEC proliferation, migration, survival, tubule formation and thus, angiogenesis, but VEGFR-2 has also been shown to be expressed by LECs (Gupta et al., 1999, Makinen et al., 2001a, Podgrabinska et al., 2002, Saharinen et al., 2004). Specifically, VEGFR-2 has been found on collecting LVs and in lymphatics undergoing active lymphangiogenesis (Jeltsch et al., 1997, Veikkola et al., 2001, Saharinen et al., 2004). VEGFR-2 is also activated by the full proteolytic products of VEGF-C and VEGF-D (Joukov et al., 1997, Achen et al., 1998). In addition, VEGFR-2 signaling has been shown to promote LV enlargement, but it does not appear to be involved in LV sprouting (Wirzenius et al., 2007). Deletion of the *Vegfr-2* gene in mice results in the absence of a functional vasculature due to the embryonic arrest in the differentiation of hematopoietic cells and ECs (Shalaby et al., 1995). Furthermore, removal of both *Vegf-a* alleles in mice results in almost a complete lack of vasculature where as the removal of a single allele is still embryonically lethal due to an arrest in vasculogenesis, demonstrating that *Vegf-a* expression is required for vascular patterning and assembly (Carmeilet et al., 1996, Ferrara, 1996a, 1996b, Oliver and Alitalo, 2005). Through VEGFR-2 signaling, VEGF-A also possesses lymphangiogenic potential; it has been shown to induce LEC proliferation, LN lymphangiogenesis and thus, tumor metastasis (Nagy et al., 2002, Hirakawa et al., 2005). Over-expression of VEGF-A promoted lymphangiogenesis at the site of wound healing and blockade of VEGFR-2 signaling prevented LV formation (Hong et al., 2004b).

Lymphangiogenesis has been associated with cancer, but lymphangiogenesis also occurs in wound healing and in response to chronic inflammation such as psoriasis, Crohn's disease, graft rejection or pathogen infection. However, it is unclear whether the new LVs are present to resolve the inflammation by removing excess extravasated fluids, protein clots and apoptotic cells or if the new LVs are a consequence of the pathology (Angeli and Randolph, 2006). Even

though lymphangiogenesis is generally perceived to be a beneficial process that provides a network of vessels to drain edematous tissues, results from several models suggest that inflammation-induced lymphangiogenesis can exacerbate the inflammation rather than controlling it. In addition, the massive expansion of the lymphatic network actually leads to defective LVs and thus decreased drainage and disease progression (Kovi et al., 1981, Kunstfeld et al., 2004, Angeli and Randolph, 2006). For example, over-expression of VEGF-A or VEGF-C results in lymphatics becoming structurally and functionally abnormal and lymphatic function is impaired (Jeltsch et al., 1997, Nagy et al., 2002, Angeli and Randolph, 2006). The chronic inflammatory response seen in psoriasis patients is mediated by VEGF-A and characterized by lymphangiogenesis and dilated lymphatics (Kunstfeld et al., 2004). Furthermore, systemic blockade of VEGF-A in animal models of psoriasis reduced skin inflammation (Schonthaler et al., 2009). It has also been proposed that inflammatory lymphangiogenesis is the primary step in the formation of tertiary lymphoid organs that organize in response to a state of chronic inflammation such as solid organ transplant rejection and the increased LV density around transplanted human kidneys undergoing rejection resembles tertiary lymphoid organs (Kerjaschki et al., 2004, Angeli and Randolph, 2006, Drayton et al., 2006, Thaunat et al., 2006). However, the mechanisms and players responsible for the induction of lymphangiogenesis in a state of inflammation are not completely understood and this field is only at its nascence compared to the study of tumor-induced lymphangiogenesis. (Angeli and Randolph, 2006, Drayton et al., 2006)

Lymphangiogenic VEGF molecules have also been able to improve lymphatic function when it is impaired. In a mouse ear model of postsurgical lymphedema, recombinant VEGF-C improved lymphatic function, increased dermal vascularity and normalized tissue architecture

resulting from chronic lymphatic insufficiency (Szuba et al., 2002). The local transfer of naked plasmid DNA encoding VEGF-C in a rabbit ear model led to a decrease in thickness and volume of lymphedema as well as an increase in lymphatic function as measured by lymphoscintigraphy (Yoon et al., 2003). These effects on lymphedema were also confirmed using a mouse tail model where VEGF-C decreased tail thickness and induced the proliferation of LECs and thus LV growth (Yoon et al., 2003). In the Chy mouse model of secondary lymphedema which exhibits chylous ascites and swollen limbs due to a lack of subcutaneous lymphatics as a result of an inactivating *Vegfr-3* mutation, virus-mediated VEGF-C gene therapy was able to generate functional lymphatics (Karkkainen et al., 2001). These results demonstrate that VEGF-C selectively promotes lymphangiogenesis which improves the lymphatic pathology associated with lymphedema. These studies also provide the support for the potential use of VEGF-C as a therapeutic agent for lymphatic pathologies.

Other molecules such as cytokines like IL-8, IL-6, IL-3 and IL-7 have also been implicated in supporting the function of LECs and LVs. The role of these cytokines in modulating lymphatic architecture emphasizes intimate interaction between the lymphatics and the immune response. For instance, IL-8 is generally thought of as a member of the α-chemokine or CXC chemokine subfamily that operates as a pro-inflammatory cytokine and a chemotactic factor for neutrophils, but it has also been shown to activate T cells as well as basophils and eosinophils (Baggiolini et al., 1994, Vitiello et al., 2004). IL-8 is secreted by a variety of cells types including monocytes/macrophages, neutrophils, T lymphocytes, fibroblasts, ECs, and epithelial cells in response to inflammatory stimuli such as lipopolysaccharide (LPS), IL-1, or TNFα (Marie et al., 1998). However, IL-8 also serves as potent angiogenic factor promoting vascularization in tumors and injured tissues (Koch et al., 1992, Vitiello et al., 2004).

Furthermore, IL-8 plays a direct role in supporting angiogenesis *in vitro* by directly enhancing EC proliferation, survival and tubule formation while inhibiting apoptosis (Koch et al., 1992, Li et al., 2003). More specifically, IL-8 has been shown to be secreted by the human monocytic cell line THP-1 as well as human PBMCs in response to LPS and this activation is mediated by MAPK ERK1/2 (Marie et al., 1998, Vitiello et al., 2004). Furthermore, inhibitors against the MAPK cascade reduce the amount of IL-8 produced by monocytes (Marie et al., 1998).

IL-6 is normally thought of as a pro-inflammatory cytokine associated with fever; however, IL-6 also acts as an angiogenic factor by promoting VEGF expression and inducing EC motility and EC proliferation (Holzinger et al., 1993, Cohen et al., 1996, Giraudo et al., 1996, Ni et al., 2004, Yao et al., 2006).

IL-7 is a pleitropic cytokine associated with the development of B and T lymphocytes or the homeostasis of peripheral T cell populations, but recent evidence indicates IL-7 also mediates lymphangiogenesis. IL-7 has been shown to induce the expression of lymphatic-specific molecules such as LYVE-1, podoplanin and Prox-1 in ECs (Al-Rawi et al., 2005). It also enhanced EC growth, migration and tubule formation via the up-regulation of VEGF-D (Al-Rawi et al., 2005). Blocking IL-7 with neutralizing antibodies or using IL-7R EC null mutants abolished these effects (Al-Rawi et al., 2005).

IL-3 is known to promote the survival, proliferation and differentiation of pluripotent hemopoietic stem cells in addition to its roles modulating the activity of mature cell lineages; it has also been shown to stimulate EC proliferation and functional activation (Brizzi et al., 1993). IL-3 has been reported to induce EC migration and tubule formation *in vitro* as well as neoangiogenesis in a murine model of Matrigel transplantation (Dentelli et al., 1999).

Finally, IL-3 plays a role in the differentiation of ECs through its ability to up-regulate the lymphatic-specific molecules podoplanin and Prox-1 in BECs (Groger et al., 2004).

1.5 Monocytes and Lymphangiogenesis

Monocytes have been shown to induce EC proliferation and migration by soluble mediators as well as cell to cell contact (Ito et al., 1997, Schubert et al., 2008). In wound healing monocyte recruitment has been correlated with EC proliferation (Taylor and Lewis, 1986). Even the number of circulating monocytes has been correlated with the induction of angiogenesis and activated monocytes secrete many soluble products involved in EC proliferation such as VEGF and IL-8 (Koch et al., 1992, Itaya et al., 2001, Heil et al., 2002, Li et al., 2002). The activation of monocytes to induce EC proliferation is mediated by the MAPK ERK1/2 pathway and chemotherapeutic inhibition of this pathway ablates monocyte-induced EC proliferation. Taken together, these data show that monocytes can play an active role in EC proliferation (Schubert et al., 2008).

Even though monocytes induce EC proliferation, the specific effect on LECs is less defined (Alitalo et al., 2005). Monocytes secrete lymphangiogenic factors such as VEGF-C and VEGF-D and VEGF-C; VEGF-C binds VEGFR-3 which is found on the surface of a subset of peripheral blood monocytes (Schoppmann et al., 2002, Salven et al., 2003). During biological events such as cancer, wound healing or inflammation, LVs proliferate and this effect can be mediated by pro-inflammatory cytokines, especially in inflammation, which induces the secretion of VEGF-C (Pullinger and Florey, 1937, Ristimaki et al., 1997, Watari et al., 2008). Inflammatory infiltrates in human kidney transplants undergoing rejection contain proliferating host lymphatics and *Mycoplasma* infection of mouse airway epithelial cells results in robust lymphangiogenesis driven by VEGF-C and VEGF-D-expressing cells (Kerjaschki et al., 2004,

Baluk et al., 2005). In addition, the inhibition of VEGFR-3 signaling resulted in the complete arrest in the growth of LVs which exaggerated edema (Baluk et al., 2005). Furthermore, in the rabbit cornea model of lymphangiogenesis which is associated with inflammation, lymphangiogenesis can be ablated by the depletion of macrophages which suggests that macrophages can mediate the formation of LVs (Cursiefen et al., 2004).

In cancer lymphangiogenesis, tumor associated macrophages (TAM) are a primary component of the inflammatory infiltrate surrounding tumors. These cells have a dual role in anti-tumor immunity; under IL-2 stimulation, they kill tumor cells, but under IFNγ and IL-12 activation they produce potent angiogenic and lymphangiogenic factors like VEGF that potentiate tumor progression (Schoppmann et al., 2002, 2005). Macrophages, recruited by VEGF-C-producing tumors, are thought to promote tumor lymphangiogenesis by first becoming VEGF-secreting cells themselves. Here, TAMs stimulate elongation of existing lymphatics associated with the tumor as well as the recruitment of more VEGF-producing cells to maintain the lymphatic network supporting tumorigenesis. Secondarily, TAM support tumorigenesis by transdifferentiating into LECs themselves and integrating into the peritumoral lymphatic network. With an increase in the complexity and size of this peritumoral lymphatic network, there is a greater potential for metastasis. (Kerjaschki, 2005)

It is thought that EC progenitors can contribute to postnatal lymphangiogenesis and there have been two potential bone marrow-derived circulating candidate cell populations identified thus far. Salven et al. described a small population of circulating CD34+CD133+VEGFR-3+ cells that possess the capacity to differentiate into mature LECs while Schoppmann et al. identified a major subpopulation of CD14+VEGFR-3+CD31+ VEGFR-2- monocytes in human blood that can be stimulated to express VEGF-C and the LEC-specific marker LYVE-1

(Schoppmann et al., 2002, Salven et al., 2003). Endothelial progenitor cells (CD34+CD133+) have been identified in human umbilical cord blood and genetic profiling only revealed a small set of genes that were differentially expressed between these endothelial progenitors and LECs suggesting related lineages for these two cell types (Nguyen et al., 2009).

Given the existence of these two cell populations *in vivo*, it is possible that postnatal lymphangiogenesis can occur through two mechanisms: the proliferation of LECs from pre-existing vessels as well as the through recruitment of bone marrow-derived EC precursors. These cells can also incorporate into the LVs to induce two types of growth: enlarge the vessel circumferentially or join at sprout tips (Adams and Alitalo, 2007). In renal transplants, circulating LEC precursors were shown to incorporate into growing LVs (Kerjaschki et al., 2004). Furthermore, healthy corneas are typically avascular, but during inflammatory conditions both blood and lymphatic vessels invade the cornea. Using this corneal transplantation model, studies have shown that inflamed corneas contain bone marrow-derived macrophages that express VEGF-C and VEGF-D mRNA (Cursiefen et al., 2004). New LVs are formed in the inflamed corneas and this is mediated by macrophages, and not DCs or cells of the adaptive immune response or blood endothelial progenitor cells (Maruyama et al., 2005). These macrophages were also able to produce tubules and up-regulate the expression of LEC-specific markers such as LYVE-1, podoplanin and Prox-1 suggesting that macrophages transdifferentiate into LECs (Maruyama et al., 2005). It should be noted that the development of the new LVs was not only from pre-existing lymphatics but also from *de novo* lymphangiogenic aggregates of recruited cells that did not have connections to existing lymphatics (Maruyama et al., 2005). Not only did donor macrophages directly integrate into the new LVs, but local depletion of macrophages resulted in the suppression of lymphangiogenesis (Maruyama et al., 2005).

Additionally, LYVE-1+ macrophages were seen to integrate into the endothelial lining of the LVs of onchocerca nodules suggesting monocytes/macrophages play a role in the neovascularization of this structure (Attout et al., 2009).

Based on these results, monocytes and macrophages support LEC function by various mechanisms. Expansion of lymphatic vasculature occurs through the proliferation of pre-existing LECs as well as the incorporation of LEC progenitor cells into the vessels. Monocytes and macrophages may serve a dual role in lymphangiogenesis by providing the soluble mediators, like VEGF-C and VEGF-D, necessary for the division of LECs in pre-existing vessels or by transdifferentiating into LECs and directly incorporating into the vessels (Kerjaschki et al., 2004).

Even though the literature suggests that monocytes/macrophages are the predominant cell type responsible for lymphangiogenesis, other cell types such as platelets, B cells, DCs and epithelial cells have lymphangiogenic potential because of their ability to secrete lymphangiogenic molecules. Upon activation, such as clotting to initiate wound healing, platelets secrete VEGF-A and VEGF-C which induce vessel formation (Wartiovaara et al., 1998, Webb et al., 1998, Brill et al., 2004). Platelets store pro- and anti-angiogenic and lymphangiogenic molecules segregated in distinct α-granules suggesting platelets play an active role in maintaining the balance between stimulating and inhibiting angiogenesis and lymphangiogenesis upon release of these factors (Italiano et al., 2008). B cells have also been shown to regulate the expansion of the lymphatic network surrounding an activated LN during an immune response (Angeli et al., 2006). Lymphangiogenesis and, thus, DC migration into the activated LN was dependent on B cells entering the LN (Angeli et al., 2006). As a result, it is hypothesized that B cells which are recruited to the activated LN during an inflammatory

response, secrete VEGF-A and stimulate the growth of LVs and high endothelial venules through VEGFR-2 signaling (Angeli and Randolph, 2006, Angeli et al., 2006). Finally, DCs possess lymphangiogenic potential; in the corneal transplantation model, the cornea becomes infiltrated with DCs and LVs. Hamrah et al. showed that there is a rapid increase in the number of VEGFR-3+ corneal DCs under inflammatory conditions and that these cells express VEGF-C (2003). Additionally, epithelial cells were identified as the primary cell source for VEGF-A during a corneal infection with herpes simplex virus-1 (Wuest and Carr, 2010). In conclusion, it appears many different cells types have been implicated in lymphangiogenesis and that these cells exhibit increased lymphangiogenic potential during periods of inflammation arguing for an intimate link between the immune response and remodeling of the lymphatic network.

1.6 Statement of Purpose

Possible candidates mediating the filarial-induced lymphangiectasia could be discovered in the ES products of the adult worms and identifying and harnessing the biological function of these factors could help to define the pathogenesis of disease. Given the residence of the adult worms in the LVs and the location of the LECs within the same microenvironment, it is critical to understand the relationship between the ES products released by the worms and the LECs that line the lumen of infected vessels. As an initial step to characterize worm proteins and familiarize ourselves with the technologies utilized to identify proteins from complex mixtures, we performed 2-dimensional SDS-PAGE coupled with mass spectrometry. In preliminary studies, we used antigens available in abundance such as crude worm extract to provide the framework and experience base for analyses of the protein constituents and the biological relevance of the ES products. It is hypothesized that the ES products released by living adult worms stimulate the host response through an interaction with LECs.

The aim of this research is to identify the effects the ES products have on LECs or other cell types involved in lymphangiogenesis and lymphangiectasia.

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CHAPTER 2

IDENTIFICATION OF ANTIGENIC *BRUGIA* ADULT WORM PROTEINS BY PEPTIDE MASS FINGERPRINTING[1]

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2.1 Abstract

With the recent completion of the *Brugia malayi* genome, proteomics offers a new resource for a deeper understanding of the biology of filarial parasites. We employed 2-dimensional (2D) gel electrophoresis followed by peptide mass fingerprinting on a matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometer to identify *Brugia* adult worm proteins, and then determined which proteins were recognized by the host humoral immune response. We identified 18 unique proteins, several of which were determined to be antigenic by immunoblot. The proteins identified here may contribute to future studies to analyze the transmission and pathogenesis of lymphatic filariasis.

2.2 Introduction

Lymphatic filariasis is caused by the parasitic nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*, which affect an estimated 120 million people in Asia, Africa, and the Americas (WHO, 1995, 2001). Adult worms live within the lymphatic vessels of the human host and release microfilariae. The microfilariae exit the lymphatic vessels and circulate in the peripheral blood to be acquired by mosquitoes during a blood meal. In the mosquito, the microfilariae mature into third-stage larvae (L3) that are infectious to humans following a subsequent blood meal. The L3 develop into the adult worms that reside in the lymphatic vessels, thereby completing the filarial life cycle.

Clinical filariasis can include hydrocoele and lymphedema/elephantiasis, but many individuals with clinical disease are antigen-negative; conversely, many infected individuals with circulating microfilariae are asymptomatic (Lammie et al., 2002). The mechanisms of filarial-associated pathology have not been clearly elucidated; both secondary bacterial infections and genetic susceptibility are thought to play major roles in the development of lymphedema. It has

been hypothesized that individuals with disease may have a genetic predisposition since pedigree studies have shown that lymphedema clusters in high risk families (Cuenco et al., 2004). While secondary bacterial infections and genetic susceptibility have been shown to contribute to the development of disease, there is also evidence that the host immune response is associated with the clinical presentation. Previous studies have shown more robust antifilarial humoral immune responses, primarily characterized by elevated IgG1, IgG2, and IgG3, in persons with clinical disease compared to those with asymptomatic microfilaremia (Baird et al., 2002, Nielsen et al., 2002b). In addition, individuals with clinical disease have heightened cell-mediated immune responses (Ottesen et al., 1977, Addiss et al., 1995, Freedman, 1998, Nutman and Kumaraswami, 2001, Nielsen et al., 2002a, 2002b). Moreover, animal models have also demonstrated a role for the immune system in the development of disease, since reconstituting *B. malayi*-infected nude mice with primed spleen cells from chronically infected mice induces the development of lymphatic lesions not seen in nude mice alone (Vickery et al., 1991). Taken together, these data suggest that the host immune system is contributing to the development of disease.

Advances in the understanding of the pathogenesis of lymphatic filariasis have been limited by the lack of knowledge regarding patterns of antigen-specific recognition. It has been hypothesized that the disease phenotype may correlate with specific antigen recognition profiles and that characterizing these profiles could be relevant to both immunopathology and immunodiagnosis of infection.

Proteomics offers a new approach to the study of filarial parasites (Bennuru et al., 2009, Hewitson et al., 2008, Moreno and Geary, 2008). With the completion of the *B. malayi* genome (Ghedin et al., 2007), the increased availability of mass spectrometry technology, and previous demonstrations of successful proteomic studies in other parasitic worms (Cheng et al., 2005,

Curwen et al., 2004, Dea-Ayuela and Bolas-Fernandez, 2005, Jefferies et al., 2001, van Balkom et al., 2005, Yatsuda et al., 2003), many novel immunogenic proteins can be identified. Furthermore, proteomics coupled with 2-dimensional immunoblotting facilitates the characterization of antigens that are potentially important targets of the host response. Others have begun to utilize these tools to characterize the immune response against the excretory-secretory products of filarial worms (Hewitson et al., 2008), but we are interested in the identification of adult antigens from crude worm extracts. Here, we describe a proteomic approach to identifying *Brugia* adult worm proteins, which may subsequently provide tools to help monitor transmission, diagnose infection, and elucidate the mechanism(s) associated with the pathogenesis of disease.

2.3 Materials and Methods

2.3.1 Parasite Material

Brugia pahangi adult worms collected from the peritoneal cavity of infected jirds, *Meriones unguiculatus*, were obtained from the NIH Filariasis Repository at the University of Georgia (Athens, Georgia). The worms were stored in phosphate-buffered saline (PBS) at -80 C until thawed. After thawing, crude worm extracts were prepared by cutting the adult worms with scissors, suspending the material in 3 ml PBS and ultrasonicating (Heat Systems-Ultrasonics, Inc., Plainview, New York; Model W-225 with standard tip) at 40% power, 20% duty cycle, pulse mode for 30 min at 4°C. After a 10 min centrifugation at 15,000 *g* at 4 C, the protein concentration of the supernatant was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Illinois). Prior to isoelectric focusing, soluble proteins (3.2 mg) were acetone precipitated overnight at -20 C. The precipitate was centrifuged for 15 min at 15,000 *g*. The supernatant was discarded and the pellet was dried for 30 min at room temperature.

2.3.2 2-Dimensional Gel Electrophoresis

The protein pellet was dissolved in 9.8 M urea (Sigma, St. Louis, Missouri), 4% 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma), 0.25% ampholytes, pH 3-10 (Bio-Rad, Hercules, California), 2 mM tributylphosphine (TBP; Bio-Rad) reducing agent and trace bromophenol blue (Bio-Rad). An immobilized pH gradient (IPG) strip (17 cm linear, pH 4-7; Bio-Rad) was loaded with the sample and left at room temperature overnight. Isoelectric focusing was performed in a Protean IEF Cell (Bio-Rad) at 20 C with the parameters set at 250 V for 15 min, 10,000 V for 3 hr and then 10,000 V until a total of 60,000 Volt-hr was reached. After isoelectric focusing, the IPG strips were stored at -80 C until SDS-PAGE separation was performed.

Proteins on the strip were reduced and alkylated in an equilibration step immediately prior to the transfer to the second dimension electrophoresis. The IPG strips were incubated in 6 ml of equilibration buffer (6 M urea, 20% SDS, 41 mM Tris, 40 mM boric acid, 20% glycerol, 0.1% bromophenol blue) containing 130 mM dithiothreitol (DTT; Sigma) for 10 min and in equilibration buffer containing 135 mM iodoacetamide (Bio-Rad) for 10 min. IPG strips were placed onto 3% stacking gels above 10-20% linear-gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 1.0 mm-thick gels in a Protean II set-up (Bio-Rad) and run at 10 mA per gel for 1 hr and then at 20 mA per gel for 5 hr at 15 C. The discontinuous buffer system used has been previously described (Moss et al., 1990). Two replicate gels were stained with Brilliant Blue G 250 Coomassie (Alfa Aesar, Ward Hill, Massachusetts) and spots of interest were manually excised for mass spectrometric analysis. The protein identifications are reported as a combination of proteins identified from both gels.

2.3.3 MALDI-ToF

Spots of interest were destained with 50% acetonitrile in 50 mM ammonium bicarbonate and digested with porcine trypsin (Promega, Madison, Wisconsin) overnight at 37 C. Peptides were extracted from the gels with 100% acetonitrile and dried by vacuum centrifugation. The tryptic peptides were desalted using C18 ZipTips (Millipore, Billerica, Massachusetts) according to the manufacturer's protocol (Millipore). After desalting, the peptides were eluted into 10 µl elution buffer containing 50% acetonitrile and 0.1% formic acid. A mixture of 1.5 µl sample plus 1 µl saturated α-cyano-4-hydroxycinnamic acid in 50% elution buffer was plated directly onto a MALDI target plate.

The target plate was loaded into a Proteomics 4700 MALDI-ToF-ToF Analyzer (Applied Biosystems, Foster City, California) and the spectra were acquired in reflector mode on positively charged ions. The modified analysis of the mass spectrometric data has been previously described (Curwen et al., 2004). In brief, raw spectrum files were opened in Data Explorer software (Applied Biosystems) and processed to remove the noise and de-isotope the peak lists so that only monoisotopic peaks remained. The peak list was converted to a text file and searched against the *B. malayi* amino acid sequence database (TIGR) (Ghedin et al., 2007) as well as the NCBI-NR database by the Mascot software program (Matrixscience, Boston, Massachusetts), which allows experimental peptide weights to be compared against known tryptic peptide fragments from the amino acid sequence of an organism. Search parameters were as follows: missed cleavages = 1; peptide tolerance = ±200 ppm; possible variable modifications = carbamidomethylation (due to the iodoacetamide treatment on cysteine residues). According to the Mascot software program, scores are reported as $-10*LOG_{10}(P)$ where P is the absolute probability and identified proteins were only considered significant if they were above the 95%

probability that they were not a random match as calculated by Mascot. Significant protein identifications by Mascot were confirmed by their predicted molecular weight and isoelectric point (pI) on the gel.

2.3.4 Study sera

Pooled sera (n = 20) were prepared from clinically and parasitologically characterized samples collected from several groups of patients as part of previous studies (Baird et al., 2002). Serum was collected from antigen-positive persons, those with lymphedema and residents who live in areas of Haiti where no transmission of filariasis is known to occur.

2.3.5 Immunoblotting

Crude worm protein (300 µg) separated by 2D-electrophoresis were transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) using a Trans Blot Plus Cell (Bio-Rad) at 100 V for 2 hr 20 min with coolant circulator for blot medium set at 0 C. The blot medium contained 20% methanol, 108 mM Tris/HCl, pH 9.18, and the immunoblot procedure has been previously described (Tsang et al., 1986, Moss et al., 1998). In brief, the transferred proteins were exposed to pooled human sera at a 1:50 dilution. Bound human antibodies were detected with a 1:1,000 dilution of biotinylated mouse anti-human IgG (clone HP6022; Invitrogen, Carlsbad, California). Streptavidin alkaline phosphatase (Invitrogen) was used at a 1:1,000 dilution to probe for bound biotinylated secondary antibodies. Bound human IgG was visualized by 5-bromo-4-chloro-3-indoyl phosphate (Sigma) as the substrate and nitro blue tetrazolium (Sigma) as the chromagen.

2.3.6 Image Analysis and Antigen Identification

Prior to spot excision, Coomassie-stained gels were electronically scanned (Hewlett-Packard Scanjet). Phoretix 2D v2004 software (Nonlinear Dynamics Ltd., Durham, North

Carolina) was used to compare the spots on the immunoblots to the spots on the gels. In brief, the gel and immunoblot images were opened in the software, the background was subtracted automatically and the proteins were landmarked. With minimal manual adjustments, the images were aligned and warped so that antigen identifications could be made. The Phoretix software uses the overall protein expression pattern, which is based on MW and pI of each protein, to align and warp the blots to match the gel spots with the corresponding spots on the immunoblot. The software also utilizes proteins that are recognized by all individuals (e.g. tropomyosin) as internal reference standards for this alignment. Moreover, the immunoblot spots were identified based solely on their corresponding position with the protein spot excised from the gel; no proteins were collected off of the immunoblot for MALDI-ToF analysis. More specifically, no proteins were removed and digested from the immunoblot itself.

2.4 Results

2.4.1 MALDI-ToF

Two-dimensional gel electrophoresis was used to separate *B. pahangi* adult worm proteins. The gel was stained with Coomassie blue and the most visually intense spots (n = 64) were excised. The spots were digested with trypsin, yielding peptide fragments that were analyzed by a MALDI-ToF mass spectrometer and searched against the *B. malayi* amino acid sequence database by the Mascot software program. A spot was considered to be positively identified only if it possessed a significant MASCOT score and migrated to a point consistent with its predicted molecular weight and pI in the 2D gel. Figure 2.1 shows the 2D profile and the protein spots that were identified from the Coomassie-stained gels. Only the spots that were positively identified as proteins are circled in Figure 2.1. Based on significant MASCOT scores, we were able to positively identify 38 proteins; however, 2 of these spots did not match their

predicted molecular weight and/or pI. Also, 1 spot (#27 in Fig. 2.1) had multiple protein identifications. As a result, a total of 36 total proteins and 18 unique proteins were positively identified (Table 2.1). To support the protein identifications, a representative example of the protein sequence coverage is seen in Figure 2.2 for the disorganized muscle protein. Proteins identified were involved in many different biological processes including metabolism, cell signaling and structural functions (Table 2.2).

2.4.2 Immunoblotting

In studies of Haitian populations living in an endemic area for lymphatic filariasis, it has been shown that persons with lymphedema and those with circulating antigen exhibit differential antibody reactivity to filarial antigens (Baird et al., 2002). We performed immunoblots to compare patterns of specific antibody responsiveness with pooled sera from antigen-positive persons and those with lymphedema (Fig. 2.3). We also probed immunoblots with sera from unexposed individuals as a negative control.

Using Phoretix software, which allows the overlay of gels and immunoblots, we analyzed gel to blot pairs to determine if there were any differences in antigen recognition associated with specific patient groups (Fig. 2.3). The intensity of antibody reactivity by immunoblots prepared with sera from lymphedema patients was greater than that of antigen-positive persons; however, the proteins recognized by both groups were similar. For example, tropomyosin, transglutaminase, the 14-3-3-like protein and galectin elicited a strong immune response from both patient groups. In contrast, HSP70 was recognized more intensely by those with lymphedema. The spot identified as disorganized muscle protein was visually present on both groups' immunoblots, but the software analysis identified disorganized muscle protein solely on the antigen-positive immunoblot.

By immunoblot, unexposed persons recognized tropomyosin (data not shown) but this reactivity could be due to a non-specific cross-reactivity resulting from infection with other helminths (Arruda and Santos, 2005).

2.5 Discussion

Proteomics can offer exciting new resources to fuel the fight against lymphatic filariasis through the discovery of new proteins, which may lead to the development of improved diagnostics and a better understanding of the host immune response. With the completion of the *B. malayi* genome (Ghedin et al., 2007), the proteome can now be characterized using mass spectrometry. Previous work in filariasis has begun to characterize the excretory-secretory products (Hewitson et al., 2008) and stage- and gender-specific proteins (Bennuru et al., 2009, Moreno and Geary, 2008), but here we utilized MALDI-ToF mass spectrometry to identify *Brugia* adult worm proteins, and coupled these results with immunoblotting to identify the antigenic proteins.

Traditionally, the search for worm antigens has been focused on screening filarial libraries; however, a proteomics approach provides an alternative, more direct method to identify a new set of antigenic proteins. In addition, previous work carried out by others has demonstrated the benefits of using proteomics to define targets of the host's immune system (Dea-Ayuela and Bolas-Fernandez, 2005, Hewitson et al., 2008, Mutapi et al., 2005, Wilson et al., 2008, Yatsuda et al., 2003). We identified several structural and housekeeping proteins such as tropomyosin, actin, and HSPs, so we were confident that we were able to identify proteins typically associated with a proteomics approach. Moreover, we were also able to identify several filarial-specific proteins that have been previously characterized due to their recognition by the host immune response.

Many of the proteins identified have been shown to play a role in the host immune response to filarial parasites. For example, fructose-1,6-bisphosphate aldolase has been shown to induce a protective immune response in a mouse chamber model of onchocerciasis, leading to approximately 50% reduction in larval survival (McCarthy et al., 2002). Another filarial protein identified in the study, the *Onchocerca volvulus* protein tropomyosin, has been reported to confer protective immunity in various rodent vaccination models inducing not only a significant reduction (48-62%) in recovery of the microfilariae from the skin, but also a 46% reduction in the recovery of adult worms (Taylor et al., 1996). In addition, mice with serum-transferable protection against microfilariae produce an abundance of antibodies specific for tropomyosin (Jenkins et al., 1998). Furthermore, there was an inverse correlation between antibody levels against tropomyosin and the microfilarial densities in the skin in human infections, suggesting the clinical relevance of the humoral response to this protein (Jenkins et al., 1998). However, it should be noted that mice immunized with the full-length tropomyosin developed potent serological responses, but this reactivity was not associated with statistically significant levels of protection to challenge with infective larvae, arguing that serologic responses may not predict vaccine efficacy (Harrison et al., 2000).

Similarly, HSP70 was initially thought to be a potential candidate to induce protection since it is recognized predominantly by endemic normal persons and it is expressed by *B. malayi* L3 exposed to 37 C which would mimic larval exposure to the mammalian host (Rothstein et al., 1989). Given the elevated serologic reactivity of endemic normals, it was thought that the immune response was targeting the larval stage and preventing the establishment of infection (Peralta et al., 1999). However, rodent immunizations with *O. volvulus* HSP70 only resulted in a modest decrease in worm burdens suggesting that no significant protection is associated with this

antigen (Peralta et al., 1999). Selkirk et al. demonstrated that antibodies from *B. malayi*-infected individuals did seem to be directed at filarial-specific epitopes of HSP70, but there was also cross-reactivity from individuals infected with *Plasmodium falciparum* and *Schistosoma mansoni*, suggesting that this molecule is not suitable for diagnostics because of extensive cross-reactivity (1989).

Other proteins identified in this the study have also been identified previously as diagnostic targets. The protein identified in Genbank as *B. malayi* antigen, also known as SXP1 (Dissanayake et al., 1994), and many homologs have been evaluated in various studies. The homologs Wb-SXP-1 (Dissanayake et al., 1994, Rao et al., 2000, Baskar et al., 2004, Lammie et al., 2004), Bm-SXP-1 (Dissanayake et al., 1994), BmSXP (Rahman et al., 2007), and the well-known Bm14 recombinant antigen characterized by Chandrashekar et al. (1994) have shown great potential for use in filarial diagnostic assays. Together, these studies have shown that detection of IgG4 antibodies to this protein by ELISA is highly sensitive and specific for both brugian and bancroftian filariasis and that there is no cross-reactivity either with *Strongyloides stercoralis* or other intestinal helminths.

The proteomics approach used in this study provides an initial catalogue of immunoreactive proteins in human filariasis, but it is important to note the limitations to the study. First, *B. pahangi* was utilized in the preparation of the material, but the mass spectrometry data was searched against the *B. malayi* amino acid sequence. We decided to use *B. pahangi* worms due to the increased availability of these parasites, since this approach required a great amount of material. However, we were then forced to rely on the high degree of homology between the *Brugia* species for our protein identifications (Maizels et al., 2001). Second, the mass spectrometry data was searched against the *Brugia* amino acid sequence as

well as the NCBI-NR database under specified parameters and the peptide mass tolerance for MALDI-ToF mass spectrometry could be considered less stringent compared to other mass spectrometric techniques, but this could be a reflection of the parasite material, the 2D gel electrophoresis approach or the decreased sensitivity of a MALDI-ToF mass spectrometer compared to other instruments. As with other proteomics studies, we were not able to identify all of the excised spots as proteins; we defined positive identification as a spot with a significant MASCOT score. The lack of positive identifications could be due to the decreased sensitivity of the 2D gel/MALDI-ToF technique, an insufficient amount of parasite material or an incomplete trypsin digestion. However, the identification of just over half of the excised spots is consistent with other 2D gel/MALDI-ToF studies published on parasites (Curwen et al., 2004, Parodi-Talice et al., 2004). Finally, we used pooled serum samples to probe our immunoblots. We decided to use pooled sera to have a representative sample for each group and to decrease the amount of individual variation, but this could have biased our results toward the null hypothesis and made the recognition profiles appear more similar. In other words, if persons in one group were more likely to recognize a particular antigen than those in another group, we would have overlooked this difference. In the future, it will also be necessary to perform more detailed analyses on individual serum samples as well as an examination on the immune recognition profiles to particular antigens by each isotype.

In conclusion, the present study has identified 18 *Brugia* proteins by MALDI-ToF mass spectrometry. The overlay of 2D gels and immunoblots by computer software allowed the identification of antigenic proteins recognized by Haitians living in an endemic area. Results showed that the initial recognition profiles between antigen-positive persons and those with lymphedema were similar, with several proteins being recognized by both groups. The

similarities between proteins recognized by the different patients groups argues for the possibility of identifying an antigen that may serve as a marker of exposure which could help monitor the transmission of filariasis in the context of ongoing elimination programs for lymphatic filariasis. Many of these antigens have not been thoroughly described and will require further characterization for potential application in diagnostics, vaccine development and chemotherapeutics. With the completion of the *B. malayi* genome, future proteomics studies will add useful information to the global effort to eliminate lymphatic filariasis.

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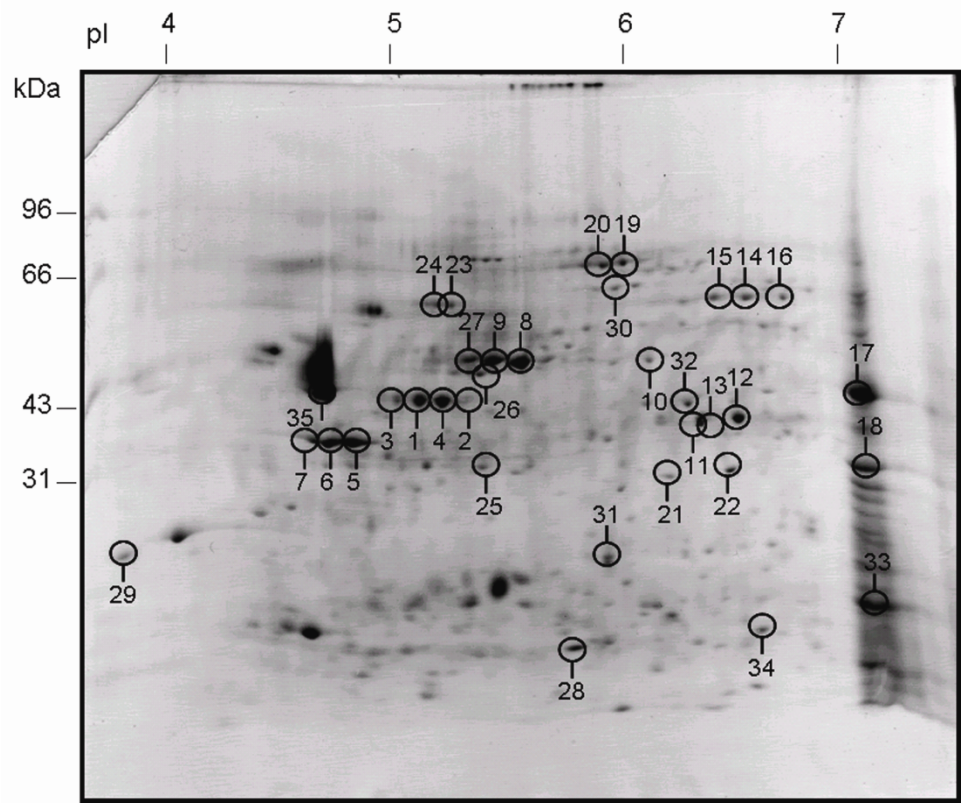
2.7 Figures and Tables

Figure 2.1. 2D gel electrophoresis of soluble *Brugia* adult worm proteins. *B. pahangi* adult worm proteins (3.2 mg) were subjected to IEF (pH 4-7) and then to a SDS-PAGE separation. The 2D gel was stained with Coomassie blue and the most visually intense spots (n = 64) were excised for MALDI-ToF analysis. Only the spots that were positively identified as proteins are numbered and listed in Table 2.1.

Figure 2.1


pI
4
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kDa
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20 19
24 23
15 14 16
27 9 8
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13 12
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Table 2.1.
Protein identifications by MALDI-ToF mass spectrometry

Unique Protein	Spot	Protein name	*Brugia* identifier from TIGR database	Theoretical MW	Theoretical pI	Mascot score
1	1	Disorganized muscle protein 1	14972.m07771	35508	4.99	105
1	2	Disorganized muscle protein 1	14972.m07771	35508	4.99	104
1	3	Disorganized muscle protein 1	14972.m07771	35508	4.99	97
1	4	Disorganized muscle protein 1	14972.m07771	35508	4.99	61
2	5	14-3-3-like protein 2	13662.m00125	29351	4.82	118
2	6	14-3-3-like protein 2	13662.m00125	29351	4.82	66
2	7	14-3-3-like protein 2	13662.m00125	29351	4.82	60
3*	8	Actin 1	14965.m00431	41777	5.30	117
3*	9	Actin 1	14965.m00431	41777	5.30	102
3*	10	Actin 1	14594.m00163	41777	5.30	71
4	11	Galectin	14731.m01012	31927	5.96	156
4	12	Galectin	14731.m01012	31927	5.96	151
4	13	Galectin	14731.m01012	31927	5.96	109
5	14	Transglutaminase	14937.m00487	56899	6.05	85
5	15	Transglutaminase	14937.m00487	56899	6.05	70
5	16	Transglutaminase	14937.m00487	56899	6.05	55
6	17	Fructose-1,6-bisphosphate aldolase 1	14176.m00093	39488	7.64	65
6	18	Fructose-1,6-bisphosphate aldolase 1	14176.m00093	39488	7.64	65
7	19	Heat shock 70kDa protein	14977.m04983	73833	5.71	60
7	20	Heat shock 70kDa protein	14977.m04983	73833	5.71	59
8	21	P27	14907.m00563	19090	6.52	62
8	22	P27	14907.m00563	19090	6.52	56
9	23	Protein disulfide isomerase	14972.m07552	54563	4.94	88
9	24	Protein disulfide isomerase	14972.m07552	54563	4.94	75
10	25	20s proteasome α5 subunit	14972.m07172	27378	5.28	91
11*	26	Actin	14258.m00140	41793	5.29	77
12*	27	Actin 2	14594.m00167	41778	5.30	64
13	28	*Brugia malayi* antigen	14975.m04515	17220	5.95	64
14	29	Calmodulin	14992.m10856	16517	4.09	72
15	27	Cytoplasmic intermediate filament protein	15463.m00018	37425	5.35	75
15	30	Cytoplasmic intermediate filament protein	15463.m00018	37425	5.35	60
16	31	DJ-1 family protein	13325.m00230	19800	5.97	55
17	32	Inorganic pyrophosphatase	14271.m00285	38880	5.91	57
18	33	Major sperm protein 2	14015.m00090	14352	7.79	81
19	34	Peptidyl-prolyl cis-trans isomerase	14990.m07926	11534	5.84	53
20	35	Tropomyosin family protein	12630.m00063	36213	4.63	58

* isoforms which may have been identified by homologous peptides

Figure 2.2. Example of peptide mass fingerprinting protein sequence coverage. The experimental peptides that matched to disorganized muscle protein (Spot 2-*Brugia* TIGR ID 14972.m07771) as reported by Mascot are shown in bold. The protein sequence coverage seen here for disorganized muscle protein (38%) is representative of the average protein sequence coverage (36.8%) for all identified proteins.

Figure 2.2

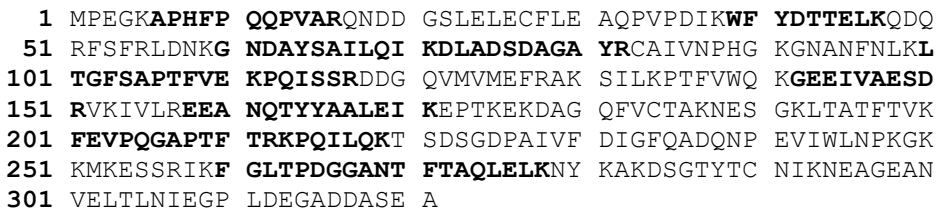

1 MPEGKAPHFP QQPVARQNDD GSLELECFLE AQPVPDIKWF YDTTELKQDQ
51 RFSFRLDNKG NDAYSAILQI KDLADSDAGA YRCAIVNPHG KGNANFNLKL
101 TGFSAPTFVE KPQISSRDDG QVMVMEFRAK SILKPTFVWQ KGEEIVAESD
151 RVKIVLREEA NQTYYAALEI KEPTKEKDAG QFVCTAKNES GKLTATFTVK
201 FEVPQGAPTF TRKPQILQKT SDSGDPAIVF DIGFQADQNP EVIWLNPKGK
251 KMKESSRIKF GLTPDGGANT FTAQLELKNY KAKDSGTYTC NIKNEAGEAN
301 VELTLNIEGP LDEGADDASE A

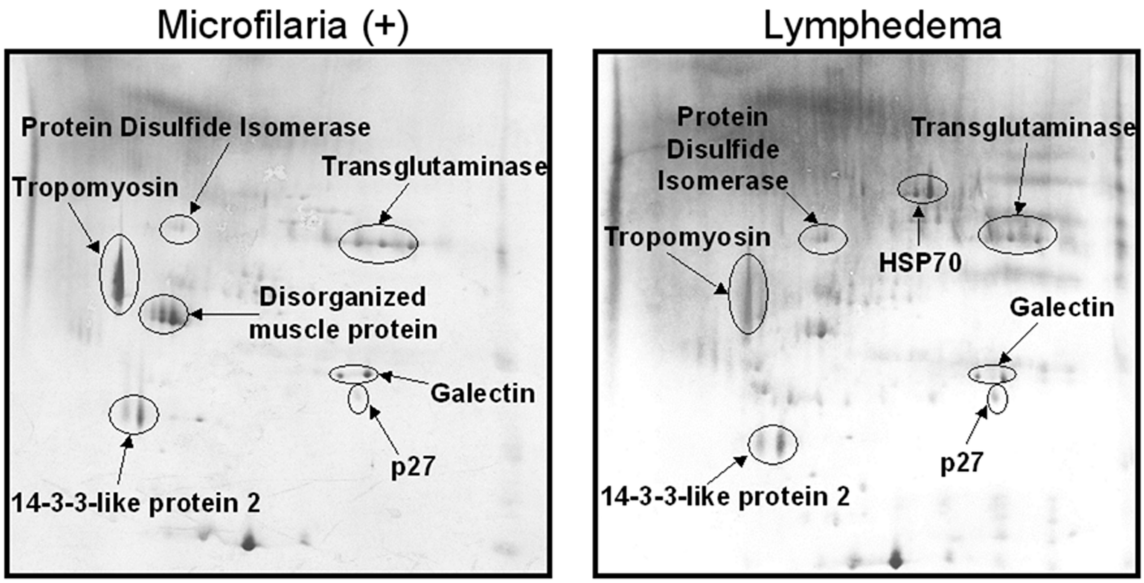
Table 2.2.
Protein identifications separated by function

Functional category	Protein name
Binding	14-3-3-like protein 2* (binds proteins)
	Calmodulin* (binds Ca ions)
	Galectin* (binds sugars)
Chaperone	HSP70
	P27
Degradation	20s alpha 5 subunit*
Electron transport	Protein disulfide isomerase*
	Tranglutaminase*
Metabolism	Fructose-1,6-bisphosphate aldolase 1
	Inorganic pyrophosphatase
Motor	Actin
	Actin 1
	Actin 2
Protein folding	Peptidyl-prolyl cis-trans isomerase
Structural activity	Cytoplasmic intermediate filament protein*
	Disorganized muscle protein 1
	Tropomyosin family protein
Unclassified	*Brugia malayi* antigen
	DJ-1 family protein
	Major sperm protein 2

* TIGR Gene Ontology classifications given priority

Figure 2.3. 2D immunoblots of microfilaria-positive persons or those with lymphedema. 2D gels containing 300 μg protein were blotted onto a membrane, probed with pooled sera from the 2 patient groups and antigenic proteins were visualized using a biotin-streptavidin AP system. The 2D gels were compared with the 2D immunoloblots using Phoretix 2D v2004 software which identified the antigenic proteins specific for each patient group.

Figure 2.3


Microfilaria (+)
Protein Disulfide Isomerase
Tropomyosin
Transglutaminase
Disorganized muscle protein
Galectin
p27
14-3-3-like protein 2
Lymphedema
Protein Disulfide Isomerase
Transglutaminase
Tropomyosin
HSP70
Galectin
p27
14-3-3-like protein 2

CHAPTER 3

CHARACTERIZATION OF *BRUGIA* EXCRETORY-SECRETORY PRODUCTS

3.1 Abstract

Adult filarial worms and the filarial excretory-secretory (ES) products they release are thought to induce lymphangiectasia in the lymphatic vessels (LV) that they inhabit. To characterize ES products, we utilized a proteomic approach with reverse phase liquid chromatography and tandem mass spectrometry to simultaneously measure both quantitative and qualitative information on ES-derived tryptic peptides. The proteomic analysis identified a complex mixture of 52 proteins associated with a variety of functions. Many of these proteins have been previously identified in the literature, but a large number are novel proteins that have not been investigated. This initial catalogue of ES proteins may lead to improved understanding of the factors mediating the pathogenesis of disease and give us a foundation to define the mechanism of lymphangiectasia.

3.2 Introduction

Lymphangiectasia or the dilation of LVs is a characteristic feature of vessels infected with filarial worms. Adult worms reside in LVs where they release microfilaria as well as ES products. Early subclinical lymphatic dilation may result in lymphatic dysfunction and thus predispose individuals to clinical lymphedema over time; however, the mechanisms involved in the development of this initial lymphangiectasia remain elusive. It has been hypothesized that the parasite is responsible for the lymphatic dilation seen in infected vessels since removal or killing of the worms by drug treatment in both humans and animal models reverses the lymphatic

dilation (Vickery et al., 1983, 1991, Shenoy et al., 2008, 2009). Lymphangiectasia is also seen in SCID mouse models suggesting that the development of disease is not necessarily a consequence of the adaptive immune response (Vincent et al., 1984, Nelson et al., 1991). In addition, even though lymphatic dilation is greatest at the site of the worm nest, lymphangiectasia is seen throughout the infected vessel and not only restricted to the site of the worm nest, arguing a soluble mediator secreted by the worm that can travel the length of the vessel induces the initial lymphatic pathology (Case et al., 1991, Amaral et al., 1994).

Filarial ES products may be important in immune evasion and the establishment of active infection as well as contributing to the development of disease, so many worm researchers have investigated the ability of these proteins to influence the immune response. Adult *Brugia* ES proteins have been shown to exhibit immunomodulatory functions by ablating antigen-driven lymphocyte responses and generating alternatively activated macrophages (Miller et al., 1991, Allen and MacDonald, 1998). The immunomodulation by helminth secreted molecules is nicely reviewed in Hewitson et al. (2009). Furthermore, filarial ES products have also been assessed for potential as diagnostic antigens; most of these initial studies utilized radiolabeling to characterize proteins recognized by microfilaremic individuals (Kaushal et al., 1982, Maizels et al., 1985, Kwan-Lim et al., 1989).

Given the presence of lymphatic dilation along the length of the infected vessel and the intimate interactions between the adult worm and the lymphatic endothelium, filarial ES products may be important in initiating lymphangiectasia. For these reasons, we wanted to identify and catalogue the ES products released by the living adult worms. We identified 52 different proteins using LC-MS. Other laboratories were performing similar studies simultaneously, so we also compared our results with concurrent filarial proteomics findings.

3.3 Material and Methods

3.3.1 Parasite Materials and Collection of ES Products

Brugia malayi adult male and female worms were collected from the peritoneal cavity of infected jirds, *Meriones unguiculatus*, that were obtained from the NIAID Filariasis Repository at the University of Georgia (Athens, GA). For the collection of ES products, 50 live adult male and female worms were cultured separately *in vitro* for 7 days at 37 C in 10mL serum-free RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Supernatants were collected and fresh medium added daily. The microfilariae were resuspended in PBS and counted using a hemacytometer to ensure worm viability. Supernatants containing the ES products were centrifuged at 6000 x g at 4 C for 10 min to remove the microfilariae and then concentrated with a Centricon filter (Millipore, Bedford, MA) to a volume of ~300 µL. This process resulted in ~670 ng/mL or 201 ng of worm protein as measured with the bicinchoninic acid protein assay (Pierce, Rockford, IL). ES products were stored at 4 C until further use. ES products were filtered using 0.45 µm Millex-HA syringe filters (Millipore, Carrigtwohill, Co. Cork, Ireland). Two biological replicates were analyzed to document similarities in protein identification by LC-MS. For stimulations with ES products, all batches of ES products were tested for endotoxin activity using the Limulus Amebocyte Lysate QCL-1000 assay (Lonza, Walkersville, MD) and ES products were only used at concentrations ≤ 0.1 eu/mL.

Adult crude worm extracts were prepared by cutting the parasites with scissors, and then the extract was suspended in 3 mL PBS and ultrasonicated (Heat Systems-Ultrasonics, Inc., Plainview, NY; Model W-225 with standard tip) at 40% power, 20% duty cycle, pulse mode for

30 min at 4 C. After a 10 min centrifugation at 15,000 x g at 4 C, the protein concentration of the supernatant was determined by BCA assay (Pierce).

3.3.2 SDS-PAGE

Concentrated ES products or crude worm proteins were mixed 1:1 with 2xSDS loading buffer, boiled for 5 min and loaded on 0.75 mm thick 12% polyacrylamide gels. For characterization of ES products secreted by males and females and across batches, 15 µL of concentrated ES products plus 15 µL of 2xSDS buffer were loaded per lane. Proteins were electrophoresed for 45 min at 160 V in a Mini PROTEAN II System (Bio-rad) and visualized by silver stain.

3.3.3 LC-MS

B. malayi ES products were prepared as described above. The proteins were first treated with 100mM aqueous ammonium bicarbonate and then with 0.2% RapiGest SF (Waters, Milford, MA). The proteins were reduced with 100 mM DTT (Sigma, St. Louis, MO) and alkylated with 300 mM iodoacetamide (Bio-Rad) for 30 min per step. Porcine trypsin (Promega) buffered in 50 mM ammonium bicarbonate was added so that the trypsin:soluble protein ratio was approximately 1:100. The digestions were incubated overnight at 37 C and sent on dry ice to collaborators where they were analyzed by tandem mass spectrometry.

The methods used to perform LC-MS by the Protein Expression System have been previously described (Silva et al., 2006). In brief, capillary liquid chromatography of tryptic peptides was performed with a Waters CapLC system using a Waters NanoEaseTM AtlantisTM C18, 300 µm x 15 cm reverse phase column. The aqueous mobile phase contained 1% acetonitrile in 0.1% formic acid while the organic mobile phase contained 80% acetonitrile in 0.1% formic acid. Samples were loaded onto the column with 6% organic mobile phase and the

peptides were eluted with a gradient of 6% to 40% organic mobile phase over 100 min at 4.4 µL/min. The column was rinsed for 10 min in 99% organic mobile phase and re-equilibrated for 20 min. All samples were analyzed in triplicate.

Mass spectrometric analysis was performed using a Waters/Micromass Q-ToF Ultima API. The mass spectrometer was operated in V-mode and the analyses were performed in positive mode ESI using a NanoLockSpray™ source. The mass spectrometer was calibrated and LC-MS data were collected for 1.85 s in an alternating, low energy (collosion energy; 10 eV) and elevated energy (collosion energy; 28-35 eV) mode of acquisition. One cycle of data was acquired every 4 s.

The data were processed and searched using ProteinLynx Global Server (PLGS) version 2.2 and as well as the NCBI-NR database by the Mascot software program (Matrixscience, Boston, MA). Protein identifications were assigned by searching the TIGR *Brugia* (Ghedin et al., 2007) and *Wolbachia* (Foster et al., 2005) amino acid sequences. Search parameters were as follows: missed cleavages = 1; peptide tolerance = ±5 ppm; possible variable modifications = carbamidomethylation. The ion detection, clustering and normalization were processed using PLGS as described (Silva et al., 2005) and if necessary, additional analysis was performed with Spotfire Decision Site 7.2 and Microsoft Excel as described (Silva et al., 2005). Identified proteins were only considered significant if they were above the 85% probability that they were not a random match.

3.3.4 Immunoblotting for *Wolbachia* Surface Protein, WSP

Crude worm protein or ES products underwent SDS-PAGE and were electrotransferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore). Transferred proteins were exposed to PBS plus 0.3% Tween throughout each immunoblot step. The membranes were

then incubated overnight at 4 C with undiluted mouse IgG2a monoclonal antibody against WSP. The membranes were washed and incubated for 1 hour with biotinylated rat anti-mouse IgG antibody (1:1000). The streptavidin alkaline phosphatase (Invitrogen) system at a 1:1000 dilution was used to probe for bound biotinylated secondary antibodies. After a final washing, bound conjugates were visualized by 5-bromo-4-chloro-3-indoyl phosphate (Sigma) as the substrate and nitro blue tetrazolium (Sigma) as the chromagen.

3.3.5 Cell Culture

Primary adult human dermal lymphatic microvascular endothelial cells (HMVEC-dLy) (cat. CC-2810T25) were purchased from Lonza Clonetics (Walkersville, MD) and maintained in the corresponding EGM-2 MV Bulletkit media (cat. CC-3202; Lonza Clonetics). Cells were cultured and split using the Clonetics Reagent Pack (cat. CC-5034). Cells were maintained according to manufacturer's instructions and used between passages 4 through 8. In addition, the human dermal microvascular endothelial cell line, HMEC-1, (Ades 1992) was also used in parallel experiments. HMEC-1 cells were obtained from the Centers for Disease Control and Prevention (CDC) and maintained in MCDB131 media containing 15% FBS, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 90 μg/mL heparin, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin).

3.3.6 Detection of NFκB Phosphorylation

Lymphatic endothelial cells or HMECs were seeded at $5x10^5$ cells/well in a 6-well plate (Costar, Corning, NY) in EGM-2MV media and grown until confluent. Cells were starved for 2.5 hours with MCDB-131 (GIBCO) containing penicillin-streptomycin heated to 37 C. Cells were stimulated for 15 min (LEC) and 30 min (HMEC) and lysed with 25 μL/mL 1M Tris-HCl (pH 7.5), 50 μL/mL 20% SDS, 925 μL/mL H_2O, 200 μg/mL ethylenediaminetetraacetic acid

(EDTA), 1 µg/mL leupeptin, 1 µg/mL pepstatin A, 200 µg/mL Pefabloc SC (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail Set II (1:100; Calbiochem, La Jolla, CA). Lysate protein concentrations were determined by BCA (Pierce); 2.5 µg (LEC) and 5 µg (HMEC) protein per lane was electrophoresed in a 12% SDS-PAGE gel and electrophoretically transferred onto Immobilon-P PVDF membranes (Millipore).

Throughout the western blotting, PBS plus 0.3% Tween was used in each step. The membranes were then incubated overnight at 4 C with rabbit anti–phospho-pNFκB (1:300; cat. 4884; Cell Signaling Technology, Danvers, MA) or mouse anti-α-tubulin (1:4000; cat. T6074; Sigma) antibodies. The membranes were washed and incubated for 1 hour with HRP-conjugated goat anti-rabbit IgG antibody (1:1000; cat. 7074; Cell Signaling Technology) or HRP-conjugated goat anti-mouse IgG antibody (1:1000; cat. 12-349; Millipore), respectively. After a final washing, the bound conjugates were visualized by 3,3'-diaminobenzidine (Sigma) as the chromagenic substrate.

3.4 Results

The ES products of filarial worms may have important biological effects in both the establishment of infection and the development of lymphedema; therefore, we wanted to define the proteins composing the secretome of filarial nematodes. Excretory-secretory products were collected from *Brugia* adult worms daily for seven days and all seven days were pooled and defined as a single batch. Initial characterization of the ES products revealed that adult females release greater quantities of protein compared to males as demonstrated by SDS-PAGE (Fig. 3.1), so female adult worms were used for all subsequent experiments. Over the seven-day collection period of ES products, adult female worms were monitored for viability by motility and microfilariae production. Microfilaria production across seven days was consistent with

previous published reports (Kwan-Lim et al., 1989) where an initial *in vitro* acclimation period was seen over the first two days, followed by a subsequent increase of microfilariae release occurring between days 4-6, and a slight decline at day 7 (Fig. 3.2); therefore, we discontinued our ES collections on day 7. Daily SDS-PAGE profiles of the ES products across all seven days appeared similar and SDS-PAGE also demonstrated consistent protein patterns across various batches (Figure 3.3A and 3.3B). The reproducible kinetics of ES production and microfilariae release across the seven-day *in vitro* cultures suggest that the proteins produced were similar to those secreted under physiologic conditions and not the result of degenerating or dying worms.

In order to identify the proteins composing the collected filarial ES products, we employed LC-MS. In this analysis we identified 52 unique proteins involved in a variety of biological processes (Table 2.1). Many of these proteins (17/52) were also identified by others labs undertaking simultaneous proteomic studies on filarial ES products as denoted in Table 2.1. Almost 20% (10/52) of the identified proteins were hypothetical. In addition, 5 out of the 52 proteins possessed a secretory signal peptide as predicted by SignalP (Bendtsen et al., 2004) suggesting secretion through the classic pathway (Table 2.1). The unexpectedly low number of proteins with a secretory signal is not uncommon based on previous reports suggesting the lack of a secretory signal is not always indicative of the secretory potential of a protein (Moreno and Geary, 2008).

Given the emerging role for the *Wolbachia* endosymbiont in filarial pathogenesis, we also searched the LC-MS peptide data against the *Wolbachia* annotated genome sequence, but we did not detect any *Wolbachia* proteins in our *Brugia* ES products (Foster et al., 2005). To confirm the absence of *Wolbachia* from worm ES products, ES products were subjected to SDS-PAGE coupled with immunoblot analysis for the presence of the *Wolbachia*-specific protein,

Wolbachia surface protein or WSP; however, WSP was not detected in filarial ES products (Fig. 3.4A). WSP was detected in crude worm extract used as a positive control (Fig. 3.4A). In addition, there was no detectable endotoxin in the ES products as measured by the Limulus Amebocyte Lysate assay (data not shown). We were still concerned the presence of *Wolbachia* in the ES products might be below the limit of detection for our assays, so we carried out additional biological testing examining the ability of *Wolbachia* within the filarial ES products to activate cellular signaling pathways known to be stimulated by the endosymbiont. *Wolbachia* has been shown to mediate it's biological effects through TLR2 ligation (Hise et al., 2007), and TLR2 stimulation leads to NFκB phosphorylation; so we stimulated multiple cell types, such as primary lymphatic endothelial cells and the well-characterized HMEC line (Ades et al., 1992) with ES products but we did not detect phosphorylation of NFκB upon ES exposure (Fig. 3.4B). TNFα was used as a positive control for the phosphorylation of NFκB but the absence of NFκB phosphorylation by ES products suggests *Wolbachia* is not present in the ES at levels that can initiate cellular signaling events.

3.5 Discussion

Recent completion and annotation of the *Brugia* genome predicted 11,515 protein coding genes with 20% of the proteins being *B. malayi*-specific (Ghedin et al., 2007). Currently the *Brugia* draft genome covers ~90% of the coding region, so we employed mass spectrometry to identify the proteins composing the filarial ES products (Hewitson et al., 2008). In our analysis we found 52 unique proteins spanning multiple biological processes. Many of these products such as leucyl aminopeptidase, a homologue of the ES-62 of *Acanthocheilonema viteae*, the Ov-16 antigen of *O. volvulus* and the *Brugia* macrophage migration inhibitory factor (Bm MIF-1) have all been well characterized in the literature.

Many of the previously characterized proteins have been given special attention due to their immunomodulatory properties. For instance, ES-62 is a phosphorylcholine-containing glycoprotein that can interfere with cell signaling resulting in the inhibition of lymphocyte proliferation and a decreased production of pro-inflammatory cytokines (Weil and Liftis, 1987, Harnett et al., 1989, 1999, 2009, Goodridge et al., 2001, 2003, Stepek et al., 2004). ES-62 can regulate DC maturation to elicit Th2-polarized responses, induce IL-10 production and inhibit the ability of macrophages to produce Th1 cytokines (Whelan et al., 2000, Goodridge et al., 2001, McInnes et al., 2003, Wilson et al., 2003). In our ES proteomic analysis, we detected the *Brugia* homologue of Ov-16 which is a cysteine protease inhibitor that suppresses immune cell proliferation that is secreted by both *A. viteae* and *O. volvulus* (Hartmann et al., 1997, Schönemeyer et al., 2001). Bm MIF-1 was also identified in the ES; the secretion of Bm MIF-1 is thought to reflect the worm's ability to promote parasite persistence by modulating the host immune response through the secretion of products that mimic host factors and induce suppressive effects (Pastrana et al., 1998, Zang et al., 2002). LL20 15 kDa ladder protein, formerly called the gp15/400 antigen, was identified in our ES analysis and this protein has been shown to be one of the major targets of IgG4 and IgE responses in lymphatic filariasis (Paxton et al., 1993, Tweedie et al., 1993, Yazdanbakhsh et al., 1995). The LL20 15 kDa ladder protein is synthesized as a 400 kDa precursor that is secreted as 15 kDa subunits which can bind lipids and may play an additional role in the sequestration of lipid mediators involved in the local immune response (Selkirk et al., 1993, Kennedy et al., 1995, 2000). γ-Glutamyltranspeptidase or Bm2325, a protein that is known to be a target for host IgE and highly recognized in patients with tropical pulmonary eosinophilia, was also detected in our ES preparation (Lobos et al.,

1996, 1997, 2003). γ-Glutamyltranspeptidase may also be involved in leukotriene synthesis and thus eosinophil recruitment (Mayatepek et al., 2004, Cheraim et al., 2008).

In our analysis we also detected the presence of molecules that have not been extensively characterized in the filarial literature such as galectin, triose phosphate isomerase (TPI), and the transthyretin family of proteins. Galectin is of particular interest as it is thought to skew the host immune response towards a Th2 phenotype and specifically bind IgE (Klion and Donelson, 1994, Motran et al., 2008, Pou-Barreto et al., 2008, Yang et al., 2008). Recently it has been demonstrated that galectin is also recognized by filarial-infected individuals as well those exhibiting clinical disease (Hewitson et al., 2008, Weinkopff et al., 2009). We also identified TPI in our ES products which is a glycolytic enzyme that catalyzes the production of glutaraldehyde-3-phosphate and is a potential vaccine candidate for schistosomiasis (Wu et al., 2005). In our analysis, we detected multiple proteins identifying to the transthyretin family of proteins. The function of transthyretin proteins is not known for LF but these proteins are found in the cerebrospinal fluid and serum where they bind thyroid hormones, especially thyroxine, and they also serve as a carrier for retinol, or vitamin A, by binding to the retinol-binding protein to create a complex and thus prevent the loss of the retinol-binding protein through kidney filtration (Prapunpoj and Leelawatwattana, 2009).

Other laboratories have carried out proteomic analyses on the filarial secretome in parallel with our study. Hewitson et al. identified 80 proteins secreted by female and male adults using a combination of approaches including gel electrophoresis coupled to MALDI-ToF/ToF and shotgun LC-MS/MS (2008) and Moreno and Geary identified 228 total filarial proteins by LC-MS/MS with 160 proteins secreted by adult females and 76 proteins secreted by microfilariae (2008). In the Moreno and Geary study, only 7 secreted proteins were shared

between adult females and microfilariae (2008). The most recent secretome analysis performed by Bennuru et al. found 852 total proteins secreted from adult males, adult females, microfilariae, L3 or L3 molting to L4 but only 157 and 457 proteins were specific for the adult female and microfilaria stages, respectively (2009). In the Bennuru study 36 proteins were shared by adult females and microfilariae (2009). Many of the same proteins were identified by our analyses as well as the other studies but the differences in the number of identified proteins could be due to variations in the stages analyzed, variations in the technology utilized and differences in the algorithms and restriction criteria when determining protein identifications. It is important to note we identified the most abundant proteins found within the adult female worm ES products according to the other secretome studies including γ-glutamyltranspeptidase, galectin, LL20 15kDa ladder antigen and TPI which has been shown to be the most abundant protein in the ES products (Hewitson et al., 2008, Moreno and Geary, 2008, Bennuru et al., 2009).

The ES products of the worms contain a mixture of proteins actively secreted by the parasite as well as molecules that are excreted as waste products and products that may diffuse or leak from the worm such as uterine fluids. Given our limited understanding of the biological importance of these proteins, it has been proposed that we need to analyze all the ES products without prejudice with respect to their physiological origin (Hewitson et al., 2009). We agree with this approach, but we extended our approach to include both the ES products from living female adult worms co-cultured with the microfilariae they are releasing *in vitro*. Given the microfilariae are released simultaneously as the ES products *in vivo* we did not discriminate between life cycle stages. However, other filarial proteomic studies did distinguish between life cycle stages so this information is available (Moreno and Geary, 2008, Bennuru et al., 2009).

The presence of rickettsial endosymbiont *Wolbachia* in the ES products remains a controversial topic in filarial biology. In our proteomic analysis of the *Brugia* ES products, we did not detect the presence of *Wolbachia*; these findings are in agreement with results from Hewitson et al. (2008) and Moreno and Geary (2008). However, a proteomics study carried out by Bennuru et al. (2009) did identify 90 *Wolbachia*-specific proteins in their filarial ES products. The analysis performed by Bennuru et al. identified several hundred more proteins than any of the other filarial proteomic approaches, suggesting that their protein identification restrictions were less stringent or that their cultures possessed dying or degenerating worms releasing *Wolbachia*. It has been hypothesized that *Wolbachia* may be important in the pathogenesis of disease but collectively these data suggest that the host is not exposed to *Wolbachia* in the worm ES products but rather upon exposure to dying or degrading worms.

Combined, these studies examining filarial ES products have dramatically increased our understanding of filarial biology but there are several limitations to our approach as well as the other filarial proteomics approaches. The current technologies identified protein constituents in the ES products and provided insight into the relative abundance of each product but these studies do not shed light on the presence of non-protein components such as carbohydrates (Hewitson et al., 2009). In addition, not all secreted products are macromolecules so we are missing information on the small molecular weight compounds such as prostacyclins and prostaglandins known to be secreted by filarial nematodes (Liu et al., 1990, Hewitson et al., 2009). These techniques may also be neglecting the presence of proteins that are expressed at low levels but which are biologically important (Hewitson et al., 2009); however, cataloguing the ES components is a crucial first step in understanding the molecules involved in filarial infection and disease.

In conclusion, we have performed mass spectrometric analysis to identify the protein constituents found within *Brugia* ES products. Now that the proteins have been catalogued, we can focus on the biological relevance of these molecules. Proteins within the ES may be involved in a wide variety of biological processes; many researchers have exploited the ES products for the roles they play in immune evasion, but we are interested in the ability of these molecules to influence the development of disease. Given the intimate interaction between the worm and the lymphatic endothelium, filarial ES products may be contributing to the development of disease by directly activating LECs or indirectly modulating LEC function through an accessory cell type. Future experiments in our lab will examine the biological effects of the filarial ES products on LECs and thus the pathogenesis of disease.

3.6 References

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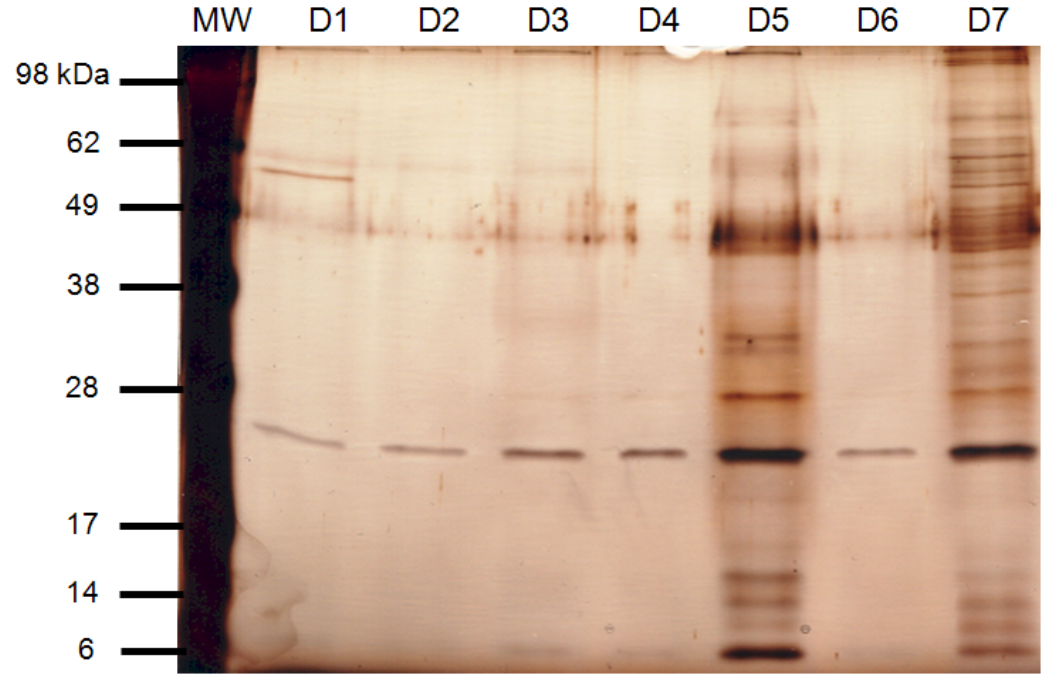
3.7 Figures and Tables

Figure 3.1. SDS-PAGE profiles of *Brugia* ES products. Adult worm ES products from (A) females and (B) males were collected daily for 7 days (D1-7) and subjected to SDS-PAGE and silver stained. MW; molecular weight marker

Figure 3.1

A. Female ES Products



B. Male ES Products

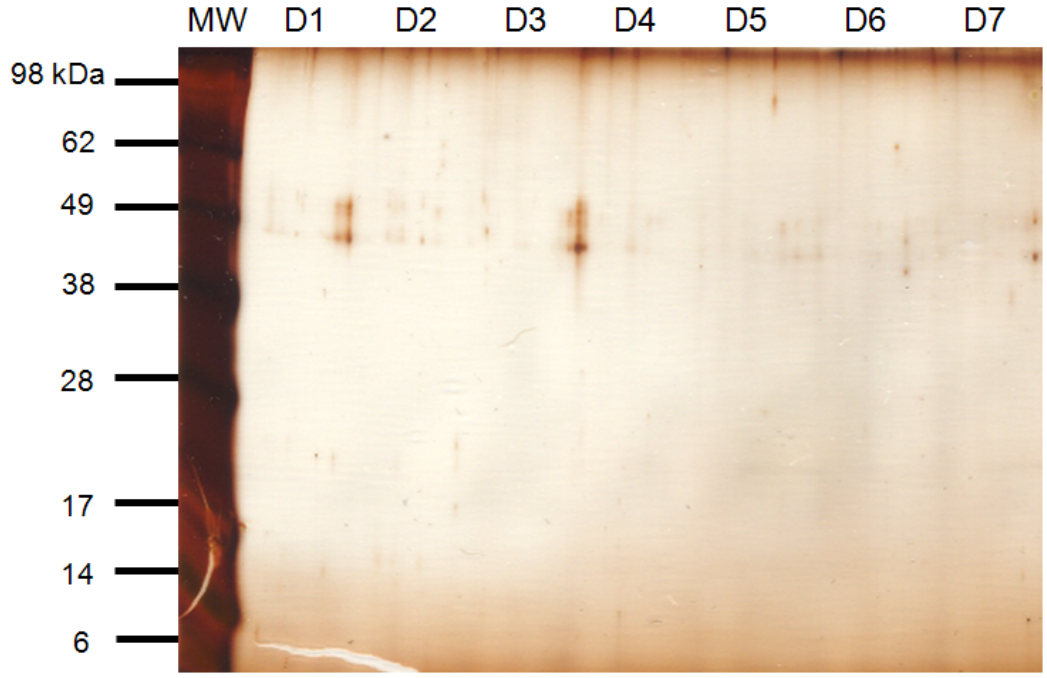


Figure 3.2. Number of microfilariae produced during *in vitro* culture of adult worms. In order to ensure female adult worm viability throughout the seven days of ES collections, worms were monitored for motility and microfilariae were counted daily using a hemacytometer.

Figure 3.2

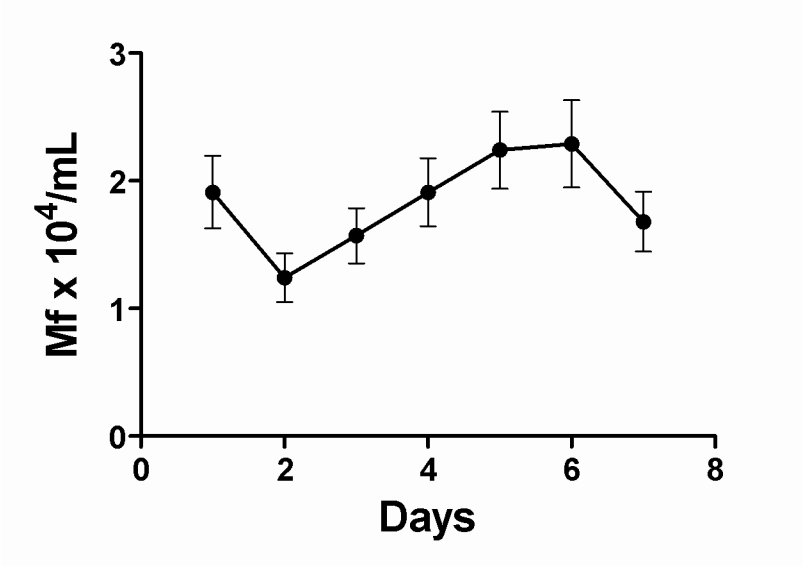
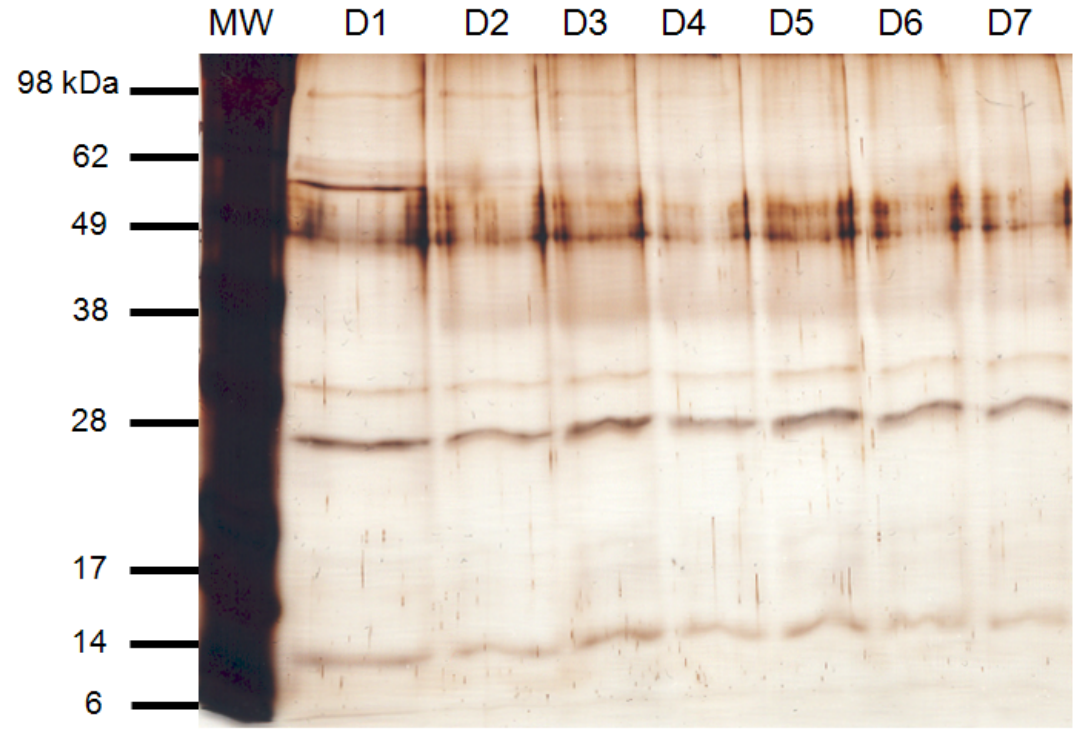


Figure 3.3. SDS-PAGE profiles of *Brugia* adult female ES products. Adult worm ES products from various batches including example batch 1 (A) and batch 2 (B) were collected daily for 7 days (D1-7) and subjected to SDS-PAGE and silver stained. Lane profiles were consistent across days and multiple batches. A batch is defined as the concentrated ES products from 50 adult female worms collected daily over 7 days and representative gels are shown for two separate batches. MW; molecular weight marker

Figure 3.3

A. Female ES Products Batch 1



B. Female ES Products Batch 2

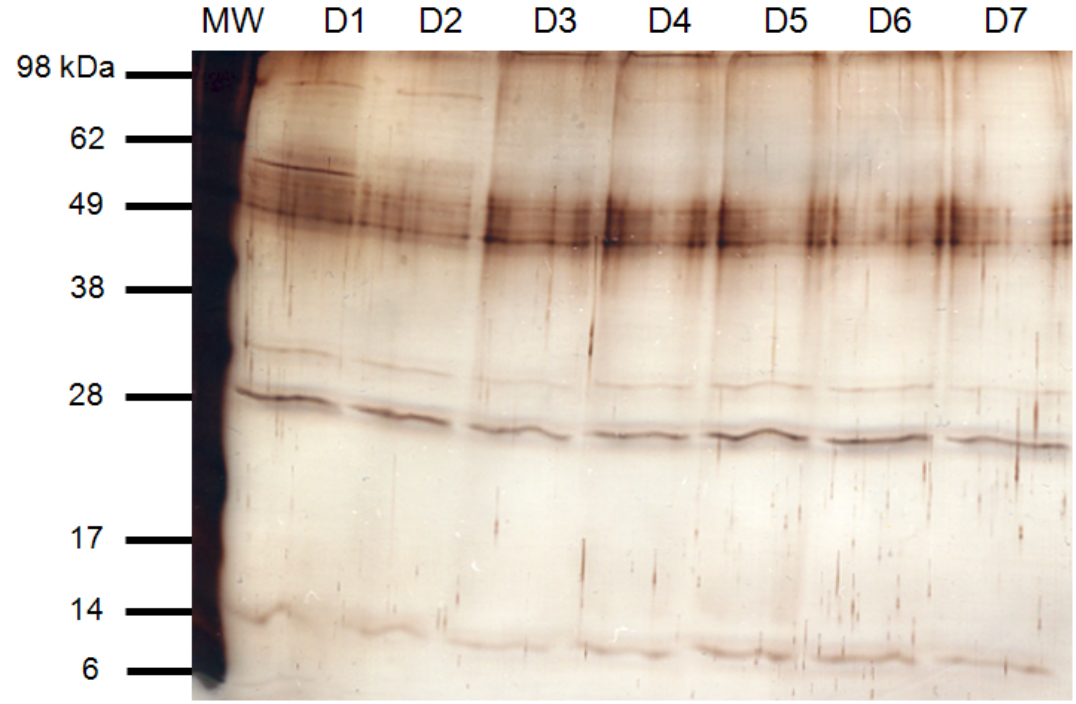


Table 3.1.
Protein identifications by LC-MS

	Protein name	*Brugia* identifier from TIGR database	Pub_locus	Score	Identified by other filarial MS studies[§]
Chaperone					
1	14-3-3-like protein 2	13662.m00125	Bm1_10970	31	A, B
Electron transport chain					
2	Hypothetical protein conserved	14528.m00116	Bm1_20620	30	
Host cytokine homologues					
3	Bm MIF-1	14930.m00337	Bm1_28435	81	A, B, C
Lectins and glycosyltransferases					
4	Galectin Bm-GAL-1	14731.m01012	Bm1_24940	224	A, B, C
5	Galectin Bm-GAL-2	14981.m02389	Bm1_46750	57	A, B
Lipid binding					
6	LL20 15 kDa ladder antigen	14992.m10973	Bm1_50995	707	A, B, C
Metabolism					
7	Fructose-bisphosphate aldolase 1	14176.m00093	Bm1_15350	90	
8	Glycosyl hydrolases family 31 protein	14972.m07829	Bm1_40580	89	A, B, C
9	Triose phosphate isomerase	14940.m00172	Bm1_29130	546	A, B, C
Protein digestion and folding					
10	γ-Glutamyltranspeptidase family protein	13531.m00015	Bm1_09950	358	A, B, C
11	Leucyl aminopeptidase*	15373.m00009	Bm1_56305	144	A, B, C
Signaling					
12	Ceramide kinase	14990.m08129	Bm1_50005	26	
13	Protein Sprint	14992.m10943	Bm1_50840	30	
14	Ser/thr-protein kinase C07A9 3	14980.m02705	Bm1_45930	35	
Structural activity					
15	Heavy neurofilament protein*	14975.m04412	Bm1_42375	95	B, C
Transcription and translation					
16	3'-5' exonuclease family protein	14975.m04472	Bm1_42650	34	
17	60S ribosomal protein L19	14961.m05302	Bm1_34025	40	
18	ATP-dependent DNA helicase RecQ family protein	14965.m00420	Bm1_34870	18	
19	Cohesin subunit A1	14992.m10991	Bm1_51090	51	
20	Dihydrouridine synthase domain containing protein	12681.m00096	Bm1_02370	31	
21	DNA-directed RNA polymerase II	12420.m00169	Bm1_00170	40	
22	Glutaminyl-tRNA synthase	13930.m00128	Bm1_12965	34	
23	Helicase	14052.m00188	Bm1_14100	68	
24	Homeobox domain containing protein	14037.m00191	Bm1_13765	26	
25	Nuclear receptor RXR	14958.m00347	Bm1_31855	25	
26	PHD-finger family protein	14271.m00291	Bm1_16985	24	
27	PWI containing protein	14992.m11048	Bm1_51370	24	
28	Snf5 homologue	14980.m02744	Bm1_46120	22	A, C

Table 3.1. *Continued*
Protein identifications by LC-MS

	Protein name	*Brugia* identifier from TIGR database	Pub_locus	Score	Identified by other filarial MS studies[§]
29	Surp module family protein	12613.m00116	Bm1_01890	24	
30	Ubiquitin*	13432.m00246	Bm1_09050	44	
Transthyretin-like family proteins					
31	Transthyretin-like family protein	13250.m00031	Bm1_06445	24	A, B, C
32	Transthyretin-like family protein*	14486.m00069	Bm1_20065	28	A, B
Unclassified					
33	Conserved hypothetical protein	13473.m00077	Bm1_09495	40	
34	Conserved hypothetical protein	13818.m00237	Bm1_12140	29	
35	HEAT repeat family protein	13538.m00201	Bm1_10000	72	
36	Hypothetical protein	14469.m00102	Bm1_19875	29	A, B, C
37	Hypothetical protein*	13987.m00026	Bm1_13345	42	C
38	Hypothetical protein	14977.m04858	Bm1_43080	21	C
39	Hypothetical protein	14702.m00398	Bm1_24075	49	
40	Hypothetical protein	14979.m04563	Bm1_45270	37	
41	Hypothetical protein	14174.m00061	Bm1_15310	26	
42	Hypothetical protein	14245.m00088	Bm1_16485	17	
43	Integrin α pat 2 precursor	12833.m00024	Bm1_03420	32	
44	Kelch motif family protein	14556.m00153	Bm1_21095	42	
45	MGC80520 protein	13311.m00337	Bm1_07295	37	
46	Ov-16 antigen precursor	14973.m02599	Bm1_41005	25	B, C
47	NADH-ubiquinone oxioreductase	14979.m04636	Bm1_45585	51	
48	Programmed cell death 6 interacting protein	14990.m07801	Bm1_48390	29	
49	Protein C20orf72 homolog	14704.m00454	Bm1_24160	27	
50	TolA protein	14704.m00455	Bm1_24165	47	
51	Variant SH3 domain containing protein	14686.m00199	Bm1_23725	31	
52	WD-repeat protein BING4	13253.m00071	Bm1_06510	19	

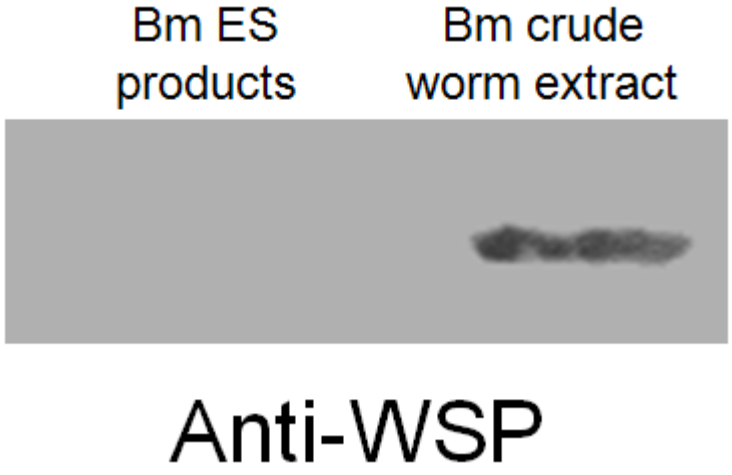
*Possesses a secretory signal according to SignalP analysis

[§]Other filarial ES proteomic studies include: A) Hewitson et al., 2008; B) Moreno and Geary, 2008; C) Bennuru et al., 2009. Proteins with no letter in this column were not identitifed in other studies.

Figure 3.4. Lack of evidence for the presence of *Wolbachia* in *Brugia* ES products. *Wolbachia* proteins were not identified by mass spectrometry so we examined *Brugia* ES products for the presence of *Wolbachia* by alternative approaches. (A) *B. malayi* ES products were subjected to SDS-PAGE followed by western blot analysis using a monoclonal antibody against WSP. Crude *Brugia* adult worm proteins were used as a positive control for the presence of *Wolbachia* as measured by the detection of WSP. (B) LECs were stimulated with *Brugia* ES products (diluted 1:10) for 0 and 15 min and lysed. 2.5 μg of cell lysate was loaded in each lane and SDS-PAGE followed by western blot analysis was carried out for the presence of phosphorylated NFκB. Multiple ES batches (ES 1 and ES 2) were used to stimulate the LECs and TNFα was used as a positive control to induce NFκB phosphorylation. The western blot seen here is also representative for the HMEC cell line that was stimulated for 30 min with ES products.

Figure 3.4

A.


Bm ES products
Bm crude worm extract
Anti-WSP

B.


0 min
Media
TNFa
ES 1
ES 2
Phosphorylated NFκB
(HMECs & LECs)

CHAPTER 4

LACK OF EVIDENCE FOR THE DIRECT ACTIVATION OF ENDOTHELIAL CELLS BY FILARIAL EXCRETORY-SECRETORY PRODUCTS

4.1 Abstract

Lymphangiectasia (dilation of the lymphatic vessel (LV)) is pathognomonic for lymphatic filariasis. In both infected humans and animal models of infection, lymphangiectasia is not restricted to the site of the worm nest, but is found along the infected vessel. These observations argue that soluble products secreted by the worm could be mediating this effect by activating the lymphatic endothelial cells (LEC) lining the vessel. Our preliminary work characterized the protein constituents of the filarial excretory-secretory products (ES) but the biological importance of these molecules remains undefined. Given the extent of LV dilation seen *in vivo*, we tested the ability of filarial ES products to activate LECs but we were not able to detect a direct effect of the ES products on the activation of LEC as assessed by a variety of approaches including cellular proliferation, cell surface molecule expression and cytokine and growth factor production (although other mediators used as positive controls did induce these effects). Collectively, these results do not support the hypothesis that ES products directly activate LECs.

4.2 Introduction

Lymphatic filariasis is a disabling disease transmitted by mosquitoes that infects more than 120 million people throughout the tropics. The infection is caused by filarial nematodes that reside in the lymphatic vasculature. There is a wide spectrum of host response phenotypes;

infected individuals often appear to be asymptomatic whereas individuals with lymphedema/elephantiasis are predominantly antigen-negative. The factors responsible for the progression of disease from infection to clinical lymphedema remain elusive. Even though infected individuals appear asymptomatic, they exhibit subclinical manifestations such as lymphangiectasia or dilated lymphatics (Freedman et al., 1994, 1995, Nutman and Kumaraswami, 2001). The parasite is thought to be responsible for alterations in the lymphatic endothelium since removal or killing of the worms reverses the dilation (Vickery et al., 1983, 1991, Shenoy et al., 2008, 2009). Furthermore, lymphangiectasia is seen in SCID mice, arguing that the adaptive immune response is not responsible for the lymphatic pathology (Vincent et al., 1984, Nelson et al., 1991). Lymphatic dilation is greatest near the site of the worm nest but it is not restricted to the site of the worm nest and is found along the length of infected vessels suggesting that a soluble product secreted by the worm may be mediating these effects (Case et al., 1991, Amaral et al., 1994).

In animal models, the lymphatics of parasitized nude mice become highly tortuous with a cuboidal endothelial lining (Vickery et al., 1985). Microscopy of dilated lymphatics of *Brugia malayi*-infected ferrets showed that dilation was greatest near the worm nests with endothelial ballooning, swelling, scarring, thickened walls and valves and thrombus formation (Case et al., 1991). These findings also support the conclusion that the presence of living adult worms and their ES products alters LV tissue. Taken together, these observations suggest that LECs are the prime targets of parasite-derived factors which initiate the development of clinical pathology (Rao et al., 1996).

We and others have previously characterized the protein constituents making up the filarial ES products released by the worm (Chapter 3, Hewitson et al., 2008, Moreno and Geary 2008, Bennuru et al., 2009) but the biological effects of these molecules have not been fully elucidated. Given the altered pathology along the length of the infected vessel and intimate relationship between the parasite and the endothelial cells lining the LVs, worm ES products may be contributing to the pathogenesis of disease. Therefore, we examined the biological effects of filarial ES products on LECs. LECs were stimulated with filarial ES products and assayed for changes in differentiation, activation and proliferation. Changes in cell surface marker expression profiles, the presence of phosphorylated cell signaling molecules, gene expression and growth factor production were used to characterize the LEC response to worm ES products. Identifying the cellular activation events in LECs induced by worm ES products will provide a greater understanding of the potential mechanisms responsible for lymphangiectasia.

4.3 Materials and Methods

4.3.1 Parasite Materials and Collection of ES Products

Brugia malayi adult female worms were collected from the peritoneal cavity of infected jirds, *Meriones unguiculatus*, that were obtained from the NIAID Filariasis Repository at the University of Georgia (Athens, GA). For the collection of ES products, 50 live adult female worms were cultured *in vitro* for 7 days at 37 C in 10mL serum-free RPMI 1640 media (GIBCO) supplemented with 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Supernatants were collected and fresh medium added daily. The microfilariae were resuspended in PBS and counted to ensure worm viability. Supernatants containing the ES products were centrifuged at 1000 x g for 10 min to remove the microfilariae and then concentrated with a Centricon filter (Millipore, Bedford, MA) to a volume of ~300 µL. ES

products were stored at 4 C until further use. Prior to cell stimulations with ES products, ES products were filtered using 0.45 μm Millex-HA syringe filters (Millipore, Carrigtwohill, Co. Cork, Ireland) and used in a dose-dependent (diluted 1:10, 1:50, 1:100) manner across various replicates and batches to maintain reproducibility. All batches of ES products were tested for endotoxin activity using the Limulus Amebocyte Lysate QCL-1000 assay (Lonza, Walkersville, MD) and ES products were only used at concentrations ≤ 0.1 eu/mL for cell stimulations.

4.3.2 Culture of ECs

Primary adult human dermal lymphatic microvascular endothelial cells (HMVEC-dLy) (cat. CC-2810T25) were purchased from Lonza Clonetics (Walkersville, MD) and maintained in EGM-2MV Bulletkit media (cat. CC-3202) from Lonza Clonetics. Cells were cultured and split using the Clonetics Reagent Pack (cat. CC-5034). Cells were maintained according to manufacturer's instructions and used between passages 4 through 8. In addition, well-characterized cell lines such as the human dermal microvascular endothelial cell line, (HMEC-1; Ades et al., 1992), the human umbilical vein endothelial cell line (HUVEC) and the bovine endothelial cell line (BOVEC) were also used in parallel experiments. hTERT-HDLEC cells were also used; these cells are composed of primary human dermal microvascular endothelial cells (HDMVEC) that were transfected with a retrovirus containing the coding region of human telomerase reverse transcriptase (hTERT) to provide a primary lymphatic endothelial cell line (hTERT-HDLEC) with an extended lifespan (Nisato et al., 2004). HMEC-1, HUVEC and BOVEC cell lines were obtained from the CDC and maintained in EBM131 media containing endothelial basal media (MCDB131; GIBCO) supplemented with 15% FBS, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 90 μg/mL heparin, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). For starvation experiments, cells were

cultured in MCDB131 media with L-glutamine and antibiotics. hTERT-HDLECs were maintained in the same EBM131 media as HMECs, but EndoGro (Vec Technologies, Rensselaer, NY) was added according to manufacturer's instructions. Cell lines were detached from flasks using 0.02% versene and 0.2% trypsin treatment.

4.3.3 Cellular Proliferation

Cells were seeded into a 96-well plate and allowed to adhere overnight. If cells were starved, cells were cultured in MCDB131 starvation media for 4 or 24 hours prior to stimulation in an array of different media and in the presence or absence of VEGF (1, 10 or 100 ng/mL) or ES products (diluted 1:10 to 1:250) for 72 hours. The cell types and culture conditions used to demonstrate EC proliferation are summarized in Table 4.1. Proliferation was assayed by adding 0.5 μCi [^{3}H] thymidine/well and measuring the incorporation of tritiated thymidine over the last 8 or 16 hours of culture using a 1205 Betaplate liquid scintillation counter (Wallac, Perkin-Elmer, Waltham, MA).

4.3.4 Detection of NFκB Phosphorylation

LECs, HMECs or HUVECs were seeded in a 6-well plate in EGM-2MV or EBM131 media and grown until confluent. Cells were starved for 2.5 hours with MCDB131 media heated to 37 C. Cells were stimulated in the warmed media for 15 min (LECs in EGM-2MV; HUVECs in EGM131) and 30 min (HMEC in EGM131) and lysed with 25 μL/mL 1M Tris-HCl (pH 7.5), 50 μL/mL 20% SDS, 925 μL/mL H_2O, 200 μg/mL ethylenediaminetetraacetic acid (EDTA), 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 200 μg/mL Pefabloc SC (Roche, Indianapolis, IN) and Phosphatase Inhibitor Cocktail Set II (1:100; Calbiochem, La Jolla, CA).

Lysate protein concentrations were determined by BCA (Pierce); 2.5 µg (LEC) and 5 or 7.5 µg (HMEC) protein per lane was separated in a 12% SDS-PAGE gel and electrophoretically transferred onto Immobilon-P PVDF membranes (Millipore).

Throughout the western blotting, PBS plus 0.3% Tween was used in each step. Following transfer, the membranes were incubated overnight at 4 C with primary antibodies purchased from Cell Signaling Technology (Danvers, MA) including rabbit anti-phospho-p44/42 MAPK (1:300; cat. 4376S), rabbit anti-phospho-pAkt (1:300; cat. 4056), rabbit anti-phospho-pNFκB p105 (1:300; cat. 4884) or rabbit anti-p44/42 MAPK (1:300; cat. 9102), rabbit anti-Akt (1:300; cat. 9272), rabbit anti-NFκB p105/p50 (1:300; cat. 3035) or mouse anti-α-tubulin (1:4000; cat. T6074; Sigma). The membranes were washed and incubated for 1 hour with HRP-conjugated goat anti-rabbit IgG antibody (1:1000; cat. 7074; Cell Signaling Technology) or HRP-conjugated goat anti-mouse IgG antibody (1:1000; cat. 12-349; Millipore), respectively. After a final washing, the bound conjugates were visualized by 3,3'-diaminobenzidine (Sigma) as the chromagenic substrate.

4.3.5 Cytokine and Growth Factor Production

LECs were plated in a 6-well plate at $3x10^5$ cells per well in 1.5 mL EGM-2MV media and the monolayer was grown until confluent. LECs were then stimulated with or without 100ng/mL LPS, 10 ng/mL TNFα or ES diluted at 1:10 for 72 hours. Cell culture supernatants were collected and stored at -80 C until further use. Supernatants were analyzed for IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFNγ, TNFα and VEGF-A by luminex technology using the Bio-Plex Pro multiplex suspension array system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Data were analyzed by the Bio-Plex Manager software version 4.1.1 and concentrations were calculated based on a standard curve derived from recombinant

cytokine standards. If the cytokine level in the sample was lower than the lowest value on the standard curve, which occurred in multiple stimulations, the lowest value of the standard curve was reported for that data point.

For the production of VEGF-C and VEGF-D, LECs were plated in a 24-well plate at $2.5x10^4$ cells per well in 500 μL EGM-2MV media and allowed to adhere for 24 hours at which point the monolayer was also confluent. LECs were starved for 24 hours in MCDB131 media and then stimulated in 1.5 mL EGM-2MV media with or without 10 ng/mL TNFα or ES diluted at 1:10 for 72hrs. Cell culture supernatants were collected and stored at -80 C until further use. VEGF-C and VEGF-D production were analyzed by the Quantikine Immunoassay kits (R&D, Minneapolis, MN) as directed by the manufacturer. All cytokine and growth factor production experiments were carried out in triplicate.

4.3.6 Flow Cytometry

LECs, HMECs or HUVECs were grown to confluence in their appropriate media and either starved for 24 hours in MCDB131 or directly stimulated in the appropriate media with or without 100ng/mL LPS, 10ng/mL TNFα or ES (diluted at 1:10 and 1:50) for 24 hours. Cells were removed from the flask by trypsinization and stained for 30 min on ice in the dark according to manufacturers' direction using unconjugated rabbit anti-human VEGFR-3 (cat. 102-PA18AG; ReliaTech, Braunschweig, Germany), mouse anti-human podoplanin (cat. 101-M40; ReliaTech) and rabbit anti-human LYVE-1 (cat. 102-PA50S; ReliaTech) primary antibodies and followed by secondary staining using FITC-labeled goat anti-rabbit (cat. 554020; BD Pharmingen) and PE-labeled goat anti-mouse antibodies (cat. 550589; BD Pharmingen). Cells were also stained using primary antibodies purchased from BD Pharmingen including conjugated PE-Cy5-labeled anti-human ICAM-1 (cat. 555512), FITC-labeled anti-human VCAM-1 (cat.

551146) and PE-Cy5-labeled anti-human E-selectin (cat. 550040). Cell events were acquired and analyzed on a BD FACScan flow cytometer (BD Biosciences, San Jose, CA).

4.4 Results

To model the intimate interaction between worm ES products and the LECs in filarial infection, an *in vitro* model system was established in which LECs were exposed to filarial ES products released by the parasite. Patients with active infection exhibit lymphangiectasia whereby LVs are dilated, but the mechanism causing this alteration is not known. The diameter of an infected LV is increased and this dilation could be a result of an increase in the number of LECs lining the lymphatic lumen, so we measured the proliferation of LECs stimulated with filarial ES products. To test this, decreasing concentrations (1:10 to 1:100) of ES products were added to LECs as well as the hTERT-HDLEC and HMEC-1 cell lines *in vitro* for various time periods and proliferation was compared to cells cultured in media alone. Under various starvation conditions and in the presence of a range of natural human or fetal bovine serum concentrations (1.25-10%) in multiple EC media (including EGM-2MV, EBM131 and basal MCDB131), we were never able to detect reproducible LEC proliferation in response to worm ES products; a representative experiment using LECs is shown in Figure 4.1.

When we did not detect increased rates of EC proliferation in response to worm ES products, we examined the ECs for earlier activation events such as the phosphorylation of cell signaling molecules. ECs including LECs, HMECs and HUVECs were stimulated with or without ES (1:10) or TNFα and the phophorylation of NFκB, Akt and MAPK ERK1/2 was compared to cells cultured in media alone at the same time points. Previous experiments established optimal time points for detection of the phosphorylated molecules in response to TNFα but we were not able to detect reproducible phosphorylation of either NFκB or Akt in

response to worm ES products. We did detect the phosphorylation of the MAPK ERK1/2 p44/42 subunit in response to worm ES products but this was a transient response that was never associated in our analyses with any downstream effects. However, the positive control TNFα did induce the phosphorylation of NFκB (Chapter 3; Fig. 3.4B), Akt and MAPK ERK1/2 (data not shown).

Cytokine and growth factor production by LECs in response to worm ES products as well as LPS and/or TNFα were also assessed in parallel with the phosphorylation studies to identify any downstream cellular activation events that might be associated with the phosphorylation of cell signaling molecules. After 72 hours of culture, IL-1β, IL-6, IFNγ and TNFα production were only detected in supernatants from LECs stimulated with LPS but not ES or TNFα; IL-8 production was similar for all experimental parameters (Fig. 4.2). Low concentrations of IL-2, IL-12, and IL-13 were detected at similar levels in cells stimulated with or without ES 1:10, LPS or TNFα compared to cells cultured in media alone (data not shown). Furthermore, IL-4, IL-5 and IL-10 production by LECs were below the detection limits of the luminex assay regardless of stimulus (data not shown). For growth factor production, similar levels of VEGF-C and VEGF-D were detected in both stimulated and unstimulated LEC supernatants after 72 hours (Fig. 4.3) and VEGF production was below the limits of detection by the luminex assay (data not shown).

Endothelial cells were also examined for changes in the expression of cell surface molecules in response to filarial ES products. ICAM-1 and VCAM-1 are adhesion molecules known to be expressed on the surface of blood vascular endothelial cells and up-regulated in response to inflammatory stimuli; therefore, we measured the expression of ICAM-1 and VCAM-1 on LECs as well as HMECs and HUVECs that were stimulated with worm ES

products for 24 hours. We did not detect an up-regulation of ICAM-1 and VCAM-1expression in any of the ECs tested in response to worm ES but we did detect an up-regulation of these adhesion molecules in response to TNFα (Fig. 4.4).

We also analyzed LECs for changes in LEC-specific surface marker expression by flow cytometry. The LEC-specific surface markers examined are summarized in Table 4.2. We did not detect and up-regulation of LYVE-1, podoplanin or VEGFR-3 surface expression on LECs that were stimulated with worm ES products or TNFα compared with the LECs cultured in media alone (data not shown).

4.5 Discussion

Our preliminary work focused on characterizing the constituents of ES products by identifying parasite proteins by LC-MS and by testing the effects of *Brugia* ES products on the direct activation of LECs. Since lymphangiectasia in microfilaremic individuals is not restricted to the site of the worm nest, soluble parasite factors may be mediating the effect *in vivo*. We originally hypothesized that the ES products of the worms were activating the lymphatic endothelium; however, we were not able to detect a direct effect of the ES products on the activation of LECs as summarized in Table 4.3. We considered that the lymphangiectasia could be due to an increase in the rate of LEC proliferation, but we did not see increased proliferation of these cells in repeated assays. In addition, the LECs did not appear to be stimulated by ES products as assessed by expression of cell surface molecules or in regard to their production of growth factors or cytokines. The positive controls in these assays induced the expected responses, so the cells were viable and capable of being activated; however, the filarial ES products did not induce detectable responses.

Some authors have proposed that lymphatic filarial parasites induce endothelial and connective tissue proliferation *in vivo* which in turn causes the thickening of the endothelium (von Lichtenberg, 1987, Buck, 1991). The cuboidal ECs seen in *B. malayi*-infected nude mice suggest that a multiplication of LECs may also contribute to lymphangiectasia (Vickery et al., 1985). However, this phenomenon could not be reproduced *in vitro* with human umbilical vein endothelial cells (Rao et al., 1996). With such contradicting results in the literature between *in vivo* and *in vitro* studies, we repeated these *in vitro* experiments with LECs, but we were still not able to detect an increase in EC proliferation in response to worm ES products under the culture conditions that we employed. In our hands, we were not able to demonstrate reproducible LEC proliferation in response to the positive control VEGF. The lack of a robust *in vitro* proliferation response to VEGF is not uncommon and many responses only exhibit <50% increase over ECs stimulated in media alone (Hirakawa et al., 2003, Hu et al., 2006, Shin et al., 2008). In general and in our cultures, the lack of LEC proliferation is most likely related to the stringency of the culture conditions required for cell growth. However, Bennuru and Nutman demonstrated microfilariae-induced LEC differentiation as measured by tubule formation suggesting further investigation needs to address the potential role of microfilariae in altering lymphatic pathology (2009). Taken together, these data suggest that EC proliferation under these culture conditions does not result from direct exposure to adult female worm ES products; however, EC proliferation may require unidentified culture conditions, other parasite stages or involve other factors, such as accessory host cells or host-derived products. The involvement of host-derived products mediating lymphangiectasia is supported by the observation that serum from infected individuals can induce LEC proliferation (Bennuru and Nutman, 2009).

Even though we did not identify a reproducible activation event induced by worm ES, we did demonstrate robust responses of LECs to the positive controls, TNFα and LPS, in multiple experiments. At the beginning of this study the appropriate positive controls and relevant concentrations suitable for LEC stimulation *in vitro* had not previously been reported. Here, we report that both TNFα and LPS are capable of stimulating LECs to activate cell signaling events, up-regulate cell surface adhesion molecules and induce growth factor and cytokine production. Since then, others have confirmed our findings; TNFα (10 ng/mL) was shown to increase ICAM-1 and VCAM-1 expression on LECs by flow cytometry (Sawa et al., 2007) and TNFα has also been reported to enhance ICAM-1 and VCAM-1 transcript levels (Pegu et al., 2008). In our hands LPS (100 ng/mL) induced the production of IL-1β, IL-6 and TNFα by LECs compared to unstimulated LECs (Fig. 4.2) and these results were later confirmed by Pegu et al. (2008). Collectively, these data argue that even though we did not detect an ES-induced activation event in LECs, our cells were viable and we had established the appropriate positive controls to induce and detect optimum responses.

In conclusion, we were not able to demonstrate a direct activation of LECs by filarial ES products. The lack of evidence for a direct activation event may be explained by the limitations of an *in vitro* culture model system. Given the longevity of filarial infections, worms can exist in LVs for years where they release soluble factors that may gradually alter the lymphatic endothelium. In our *in vitro* cultures, we were only carried out the LEC-stimulations with ES products for 72 hours; this may not be enough time to recreate the effects seen in infected vessels. The negative results may also suggest that a more complicated network is established between the parasite and the host.

These data lead to the hypothesis that LECs are not directly activated by worm ES products, but may be indirectly activated through an accessory cell type that may lead to the activation of the lymphatic endothelium.

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4.7 Figures and Tables

Table 4.1. *In vitro* cell culture conditions for EC proliferation experiments

Cell type	Media used for 72 hour stimulation	Cell No. (range)	Serum (range)	Starvation time in MCDB131 (serum-free)
BOVEC	EBM131 or MCDB131	$4x10^3$-$2x10^4$	0 or 15% FBS	24 hours
HMEC	EBM131 or MCDB131	$4x10^3$-$2x10^4$	0 or 15% FBS	No starvation; 4 or 24 hour starvation
hTERT-HDLEC	EBM131 + EndoGro	$1x10^4$	15% FBS	No starvation
LEC	MCDB131 or EGM-2MV	$2x10^3$-$5x10^4$	1.25-10% and 15% FBS 1.25-10% NHS	No starvation; 4 or 24 hour starvation

Figure 4.1. Proliferation of LECs in response to filarial ES products. $5x10^4$ LECs were plated in a 96 well plate in EBM-2MV and allowed to adhere for 24 hours. Cells were starved for 24 hours in MCBD media and then stimulated with or without VEGF (10 ng/mL) or a dilution series of filarial ES products from 1:10 to 1:100 in MCDB media containing 5% NHS for 72 hours. Thymidine incorporation was measured. Data seen here is one representative LEC proliferation experiment. Various cell lines, cell numbers, starvation conditions, media types and serum types were utilized for optimization of this assay but we never detected reproducible proliferation rates in response to ES products.

Figure 4.1

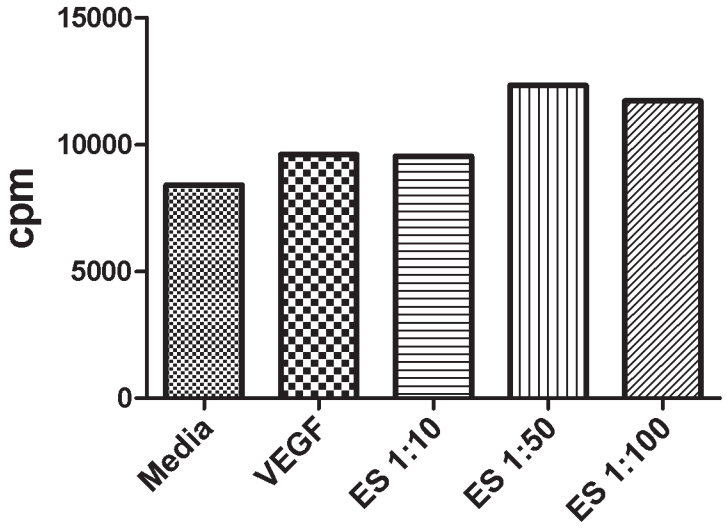


Figure 4.2. Cytokine production by LECs in response to filarial ES products. $3x10^5$ LECs were plated in EGM-2MV media and allowed to come to confluence. Cells were then stimulated in EGM-2MV media with or without ES (1:10), LPS (100 ng/mL) or TNFα (10 ng/mL) for 72 hrs and supernatants were harvested and assessed for cytokine production including IL-1β (A), TNFα (B), IL-8 (C), IL-6 (D) and IFNγ (E) by luminex bead technology. Experiments were completed in triplicate and this figure includes representative data from one experiment.

Figure 4.2

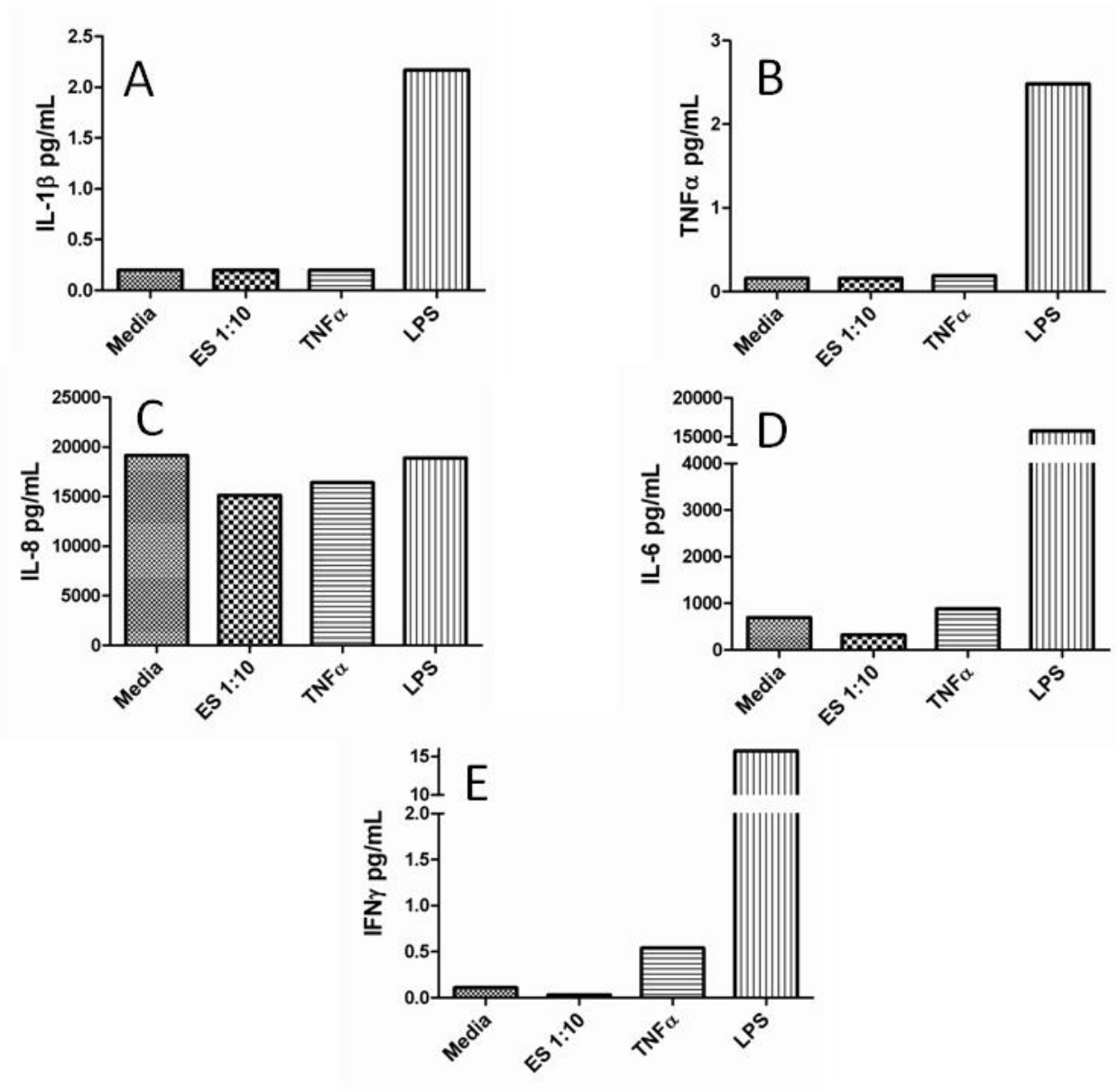
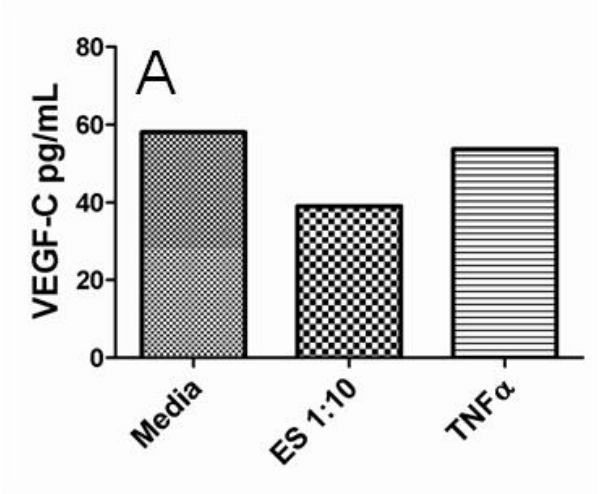

A
IL-1β pg/mL
2.5
2.0
1.5
1.0
0.5
0.0
Media
ES 1:10
TNFα
LPS
B
TNFα pg/mL
3
2
1
0
Media
ES 1:10
TNFα
LPS
C
IL-8 pg/mL
25000
20000
15000
10000
5000
0
Media
ES 1:10
TNFα
LPS
D
IL-6 pg/mL
20000
15000
4000
3000
2000
1000
0
Media
ES 1:10
TNFα
LPS
E
IFNγ pg/mL
15
10
2.0
1.5
1.0
0.5
0.0
Media
ES 1:10
TNFα
LPS

Figure 4.3. Growth factor production by LECs in response to filarial ES products. $2x10^4$ LECs were plated in EGM-2MV media and allowed to adhere for 24 hours. Cells were then starved for 24 hours in MCDB-131 media prior to stimulation for 24 hours in EGM-2MV media with or without ES (1:10) or TNFα (10 ng/mL). Supernatants were harvested and assessed for growth factor production including VEGF-C (A) and VEGF-D (B) by ELISA. Experiments were completed in triplicate and this figure includes representative data from one experiment.

Figure 4.3


A
VEGF-C pg/mL
80
60
40
20
0
Media
ES 1:10
TNFα

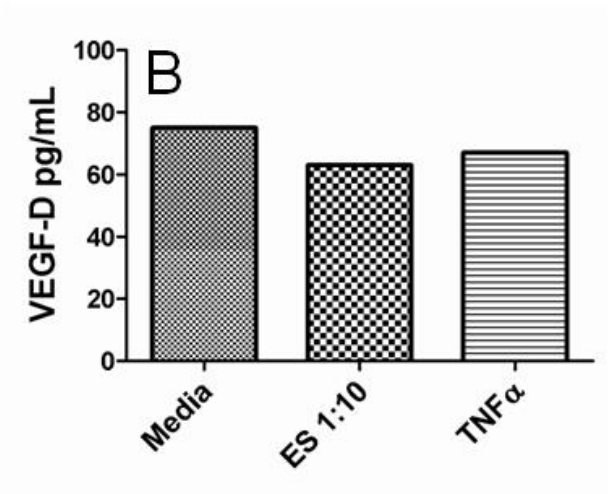

B
VEGF-D pg/mL
100
80
60
40
20
0
Media
ES 1:10
TNFα

Figure 4.4. Surface expression of adhesion molecules in response to worm ES products. LECs were grown to confluence in EGM-2MV media then cells were stimulated for 24 hours in EGM-2MV media with or without ES (1:10) or TNFα (10 ng/mL). LECs were harvested and subjected to flow cytometry analysis for the expression of ICAM-1 or VCAM-1. This figure includes representative data from one experiment.

Figure 4.4

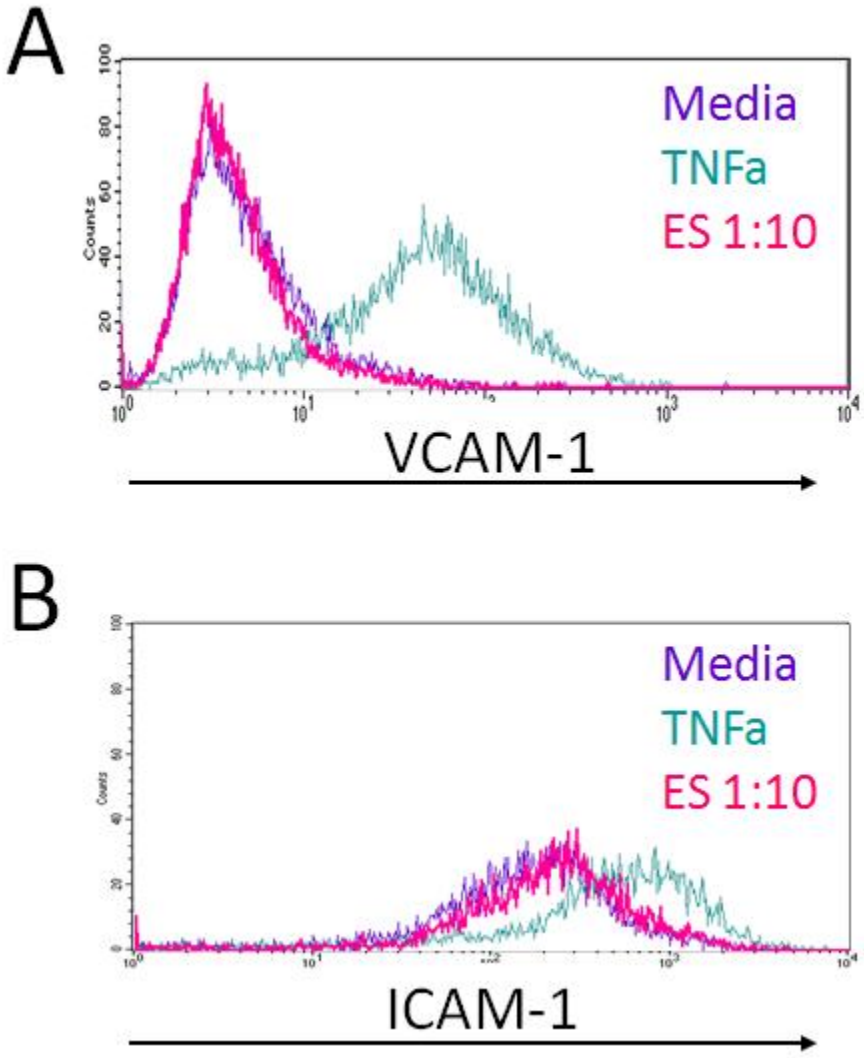


Table 4.2. Summary of lymphatic endothelial cell surface molecules

LEC surface molecule	Description & function
LYVE-1	lymphatic endothelial receptor for the ECM/lymphatic fluid glycosaminoglycan hyaluronan (Banerji et al., 1999, Nisato et al., 2004)
Podoplanin	glomerular podocyte transmembrane mucoprotein playing a role in LEC migration, adhesion and tube formation (Wetterwald et al., 1996, Breiteneder-Geleff et al., 1999, Kriehuber et al., 2001, Petrova et al., 2002, Hirakawa et al., 2003, Schacht et al., 2003, Hong et al., 2004, Nisato et al., 2004)
VEGFR-3	transmembrane tyrosine kinase receptor for VEGF-C and VEGF-D (Kaipainen et al., 1995, Nisato et al., 2004); murine mutation embryonically lethal, but skin-specific overexpression of VEGFR-3 in transgenic mice is sufficient to promote lymphangiogenesis or the development of new lymph vessels (Veikkola et al., 2001)
CD106 (VCAM-1)	adhesion molecule that binds the integrin VLA-4
CD54 (ICAM-1)	adhesion molecule that binds the integrin LFA-1
CD62E (E-selectin)	adhesion molecule that binds sialyated Lewis X and related glycans on glycoproteins

Table 4.3. Incubation of LECs with *Brugia* ES fails to induce cellular activation as measured by a variety of parameters.

Activation Event	Measurement	Cell Type
Proliferation	Thymidine incorporation	HMEC, hTERT-hdLEC[c], BOVEC or LEC
Cell signaling	Western blot for: MAPK ERK1/2, Akt, NFκB	HMEC, HUVEC, LEC[d]
Cell surface molecule expression[a,b]	Flow cytometry for: ICAM-1, VCAM-1, E-selectin, VEGFR-3, LYVE-1, podoplanin or Prox-1	HMEC, HUVEC (VCAM-1 only) or LEC
Growth factor production[a,b]	ELISA for: VEGF (VEGF-A), VEGF-C or VEGF-D	HMEC or LEC
Cytokine production[a,b]	Bioplex for: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p, IL-13, IFNγ, TNFα	LEC

[a]LPS (100 ng/ml) used as a positive control; [b]TNFα (10 ng/mL) used as a positive control; [c] hTERT-hdLEC (Nisato et al., 2004);
[d]Phosphorylation of MAPK observed in LECs but not other cell types.

CHAPTER 5

MOLECULAR MECHANISMS OF LYMPHANGIECTASIA IN LYMPHATIC FILARIASIS[1]

[1]Weinkopff, T., R. Eversole, C. Mackenzie, P. Lammie. 2010. To be submitted to *The American Journal of Pathology*.

5.1 Abstract

Lymphatic filariasis is caused by the parasitic nematodes *Wuchereria bancrofti* and *Brugia malayi* which infect over 120 million people worldwide. Most microfilaria-positive (Mf+) individuals appear asymptomatic, but exhibit subclinical manifestations such as lymphangiectasia. We hypothesized that the excretory-secretory products (ES) of the worms activate the lymphatic endothelium, but initial experiments suggested that the ES did not directly activate lymphatic endothelial cells (LEC). Monocytes have been shown to play a role in lymphangiogenesis by secreting soluble factors; so we hypothesized that monocytes were involved in filarial lymphatic pathology. We analyzed the production of IL-8, IL-6 and VEGF-A by peripheral blood mononuclear cells (PBMC) from naïve donors following stimulation with filarial ES as these factors can support endothelial proliferation and function. ES-stimulated PBMCs produced significantly increased levels of IL-8, IL-6 and VEGF-A compared to cells cultured in medium alone. CD14+ monocytes appear to be the primary producers of IL-8 and VEGF-A, but not IL-6. Furthermore, IL-8, IL-6 and VEGF-A induced *in vitro* tubule formation in LEC Matrigel cultures. Matrigel plugs that incorporated IL-8, IL-6 or VEGF-A were implanted *in vivo* and stimulated lymphangiogenesis. Collectively, these data support the hypothesis that monocytes may modulate lymphatic function through the secretion of soluble factors to encourage vessel growth and when exposed to ES products may contribute to the pathogenesis of filarial disease.

5.2 Introduction

The lymphatic system consists of a series of vessels that are lined by endothelial cells and transport fluids. The lymphatics serve several functions including immune

surveillance and fat absorption, but their primary function is to drain excess interstitial fluids to prevent tissue swelling. Blood flows through capillaries and into adjacent tissues and then exits through veins; however, about 10% of the fluid filtered by the capillaries gets caught in the tissues. About 3 liters of fluid per day remain in the tissues and without proper drainage and transport to the circulatory system by the lymphatic system, this fluid accumulation could be life-threatening (Tortora and Grabowski, 2003).

Lymphangiectasia is a condition in which lymphatic vessels are dilated and this pathology is often associated with the development of lymphedema. As lymphatic vessels become dilated, lymphatic fluid becomes stagnant and leaks back into the surrounding interstitium. Lymphatic dilation may result from a variety of causes including genetic mutations in FOXC2 or VEGFR-3, trauma and cancer-related treatments such as lymphadenectomy, but most of the lymphatic pathology seen worldwide is associated with the filarial worm parasites, *Wuchereria bancrofti* and *Brugia malayi*.

An estimated 120 million people worldwide are infected by filarial parasites (Ottesen et al., 1997). The adult worms reside in lymphatic vessels of the human host where they release a smaller microfilariae stage that is transmitted to mosquitoes. Lymphatic filariasis is an infection with varying degrees of clinical pathology. Individuals can exhibit overt clinical symptoms such as lymphedema and hydrocele or asymptomatic microfilaremia. Asymptomatic microfilaremic individuals do not appear to display any clinical manifestations, but they do present with hidden subclinical complications (Ottesen et al., 1997, Nutman and Kumaraswami, 2001) such as dilated and tortuous lymphatics (Freedman et al., 1994, 1995) and scrotal lymphangiectasia in

men (Noroes et al., 1996a, 1996b). Ultrasonographic examination of the scrotal region of 14 asymptomatic Brazilians revealed that 50% of microfilaremic individuals demonstrated lymphatic dilation and tortuosity (Amaral et al., 1994). In microfilaremic individuals, abnormal lymphatics are present in 69% of limbs by static lymphoscintigraphy and in 100% of limbs by dynamic flow lymphoscintigraphy, which are sensitive indicators of lymphatic dysfunction (Freedman et al., 1994, 1995; Dissanayake et al., 1995). In addition, studies on superficial skin punch biopsies have revealed that 78% and 68% of limbs from patients with clinical pathology and asymptomatic microfilaremia, respectively, contained lymphatic vessels that were abnormally dilated (Freedman et al., 1995, 1998). More recently, it was also demonstrated that children as young as three years of age can present with lymphangiectasia as measured by lymphoscintigraphy suggesting subclinical pathology can occur at a very early age (Shenoy et al., 2008).

The causes for the lymphatic dilation in filarial-infected individuals remain unknown but lymphangiectasia is seen in SCID mice infected with *Brugia* suggesting that the worm and/or innate mechanisms, and not the host's adaptive immune system, are involved in the development of lymphatic dilation (Vincent et al., 1984, Nelson et al., 1991). Furthermore, the dilation can be reversed in nude mice by removing or killing the adult worms (Vickery et al., 1983, 1991). Importantly, in children, Shenoy et al. showed a reduction in lymphatic dilation occurred after worm death induced by DEC treatment (Shenoy et al., 2008, 2009). Lymphangiectasia is not restricted to the site of the worm nest, but is found along the length of the infected vessel (Amaral et al., 1994) arguing that a soluble factor secreted by the worm, that can travel the length of the vessel, is

responsible for the altered lymphatic pathology. Microscopy of dilated lymphatics of *B. malayi*-infected ferrets showed that dilation was greatest near the worm nests (Case et al., 1991) supporting the conclusion that the presence of living adult worms and their ES products is associated with the alteration of the lymphatic vasculature.

The molecular mechanisms associated with the induction of lymphatic disease are being characterized. The molecules involved in the proliferation and maintenance of endothelial cells (EC) are a family of growth factors known as the vascular endothelial growth factors (VEGF) as well as cytokines such as IL-3, IL-6, IL-7 and IL-8 (Koch et al., 1992, Brizzi et al., 1993, Holzinger et al., 1993, Al-Rawi et al., 2005, Dentelli et al., 1999, Li et al., 2003, Yao et al., 2006, Adams and Alitalo, 2007). Molecules such as VEGF-A, VEGF-C and VEGF-D and their VEGF receptors, like VEGFR-3 which binds VEGF-C and VEGF-D and VEGFR-2 which can bind VEGF-A, VEGF-C and VEGF-D, have all been shown to support LEC proliferation, migration, survival and tubule formation; therefore, these molecules are potent regulators of lymphangiogenesis (Joukov et al., 1996, 1997, Lee et al., 1996, Jeltsch et al., 1997, Oh et al., 1997, Achen et al., 1998, Enholm et al., 2001, Makinen et al., 2001a, 2001b, Veikkola et al., 2001, Byzova et al., 2002, Kubo et al., 2002, Nagy et al., 2002, Podgrabinska et al., 2002, Rissanen et al., 2003, Nisato et al., 2004, Hirakawa et al., 2005, Wuest and Carr, 2010). Debrah et al. showed that plasma levels of the lymphangiogenic factor VEGF-C are significantly elevated in infected individuals as well as individuals with filarial lymphedema (2006). Elevated plasma levels of VEGF-A were also seen in individuals with hydrocele (Debrah et al., 2007).

Furthermore, human infection with the filarid, *Onchocerca volvulus*, induced lymphangiogenesis and this neovascularization of the nodule was associated with the expression of lymphangiogenic molecules such as VEGF-C (Attout et al., 2009).

Monocytes/macrophages appear to be the predominant producers of the VEGFs and the presence of monocytes/macrophages has been correlated with lymphangiogenesis (Cursiefen et al., 2004, Maruyama et al., 2005). These cells can play two major roles in the formation of new lymphatic vessels: they can produce lymphangiogenic molecules which support the expansion of the lymphatic vasculature or they can transdifferentiate into LEC themselves and integrate into the lymphatic network (Maruyama et al., 2005). In human onchocerca nodules, some mononuclear cells expressed both the macrophage marker, MAC-1, and the lymphatic-specific marker, LYVE-1, and these double-positive cells were integrated into the lymphatic endothelium (Attout et al., 2009).

Given the potential role of VEGFs and cytokines in the lymphangiectasia seen in filarial-infected individuals, we wanted to determine the ability of the worm ES products to induce the production of such lymphangiogenic molecules by host peripheral blood cells. We also wanted to identify the cell type responsible for the production of these lymphangiogenic molecules. Our results indicate that worm ES products can induce the production of VEGF-A as well as IL-8 and IL-6 and that CD14+ monocytes are the primary producers of both VEGF-A and IL-8. In addition, LEC tubule formation *in vitro* and *in vivo* was stimulated by VEGF-A, IL-6 and IL-8 compared to media alone. These data suggest that worm ES products are able to induce the production of lymphangiogenic molecules and that these molecules can influence the characteristics and behavior of LEC.

5.3 Materials and Methods

5.3.1 Parasite Materials and Collection of ES Products

Brugia malayi adult female worms were collected from the peritoneal cavity of infected jirds, *Meriones unguiculatus*, that were obtained from the NIAID Filariasis Repository at the University of Georgia (Athens, GA). For the collection of ES products, 50 live adult female worms were cultured *in vitro* for 7 days at 37 C in 10mL serum-free RPMI 1640 media (GIBCO) supplemented with 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Supernatants were collected and fresh medium added daily. The microfilariae were resuspended in PBS and counted to ensure worm viability. Supernatants containing the ES products were centrifuged at 1000 x g for 10 min to remove the microfilariae and then concentrated with a Centricon filter (Millipore, Bedford, MA) to a volume of ~300 µL. This process resulted in ~670 ng/mL or 201ng of worm protein. ES products were stored at 4 C until further use. Male worms were not used because they do not secrete the same quantity of protein material as females. Prior to cell stimulations with ES products, ES products were filtered using 0.45 µm Millex-HA syringe filters (Millipore, Carrigtwohill, Co. Cork, Ireland) and used in a dose-dependent (diluted at 1:10, 1:50) manner across various replicates and batches. A batch is defined as a specimen containing the concentrated ES products from 50 female worms over one week pooled together. All batches of ES products were tested for endotoxin activity using the Limulus Amebocyte Lysate QCL-1000 assay (Lonza, Walkersville, MD) and ES products were only used at concentrations ≤ 0.1 eu/mL for cell stimulations.

5.3.2 Isolation of Peripheral Blood Mononuclear Cells

Human PBMCs were isolated using lymphocyte separation media (MP Biomedicals, Solon, OH) as directed by the manufacturer. In brief, blood was collected from normal healthy donors by venipuncture in 10mL ethylenediaminetetraacetic acid (EDTA) Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), centrifuged for 10 min at 1000 x g at 4 C and the buffy coat was removed, resuspended in 2 mL RPMI 1640 media supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO), 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and layered over 3 mL lymphocyte separation media. Cells were centrifuged for 30 min at 1000 x g at 4 C, the buffy coat was removed, washed and cells were counted using a hemacytometer.

5.3.3 Isolation of CD14+ Monocytes

Human CD14+ monocytes were enriched from PBMCs using CD14+ MACS technology (Miltenyi Biotec, Auburn, CA) as directed by manufacturer. Briefly, PBMCs were resuspended in MACS column buffer, CD14+ MicroBeads were added and incubated with the cells for 15 min on ice. Cells were washed twice in column buffer and loaded onto the MACS MS separation column for positive selection. Trapped CD14+ cells were eluted from the column and the CD14+ monocyte isolation was confirmed by flow cytometry using mouse anti-human CD14+ PE (BD Pharmingen, San Jose, CA) and CD14+ cells were routinely enriched >94-98%.

5.3.4 Culture of LECs

Human dermal lymphatic microvascular endothelial cells (HMVEC-dLy) were purchased from Clonetics (Lonza) and maintained in EBM-2 basal media supplemented with EGM-2 MV SingleQuots (Lonza) according to manufacturer's instructions. Cells were used from passage 4-8.

5.3.5 Production of Lymphangiogenic Factors by PBMCs and CD14+ Monocytes

Cells were plated at $1x10^6$ PBMCs or $5x10^5$ CD14+ cells in 500 μL RPMI 1640 media (GIBCO) supplemented with 10% FBS, 2 mM L-glutamine and antibiotics and stimulated with or without 100ng/mL LPS or ES diluted at 1:10, 1:50 for 72hrs. Cell culture supernatants were collected and analyzed for IL-3, IL-6, IL-7, IL-8 and VEGF-A by luminex technology using the Bio-Plex Pro multiplex suspension array system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. In short, cell culture supernatants were incubated with fluorescently-labeled coupled magnetic beads for 30 min at room temperature (RT) on a shaker in a 96 well filter plate. Magnetic beads were washed 3 times and incubated with a multiplex mixture of the detection antibodies for 30 min at RT on a shaker. The beads were washed 3 times and incubated with streptavidin-PE for 10 min at RT on a shaker. The beads were washed 3 more times, resuspended in 125 μL of assay buffer, shaken and the plate was read using low PMT voltage settings. Data were analyzed by the Bio-Plex Manager software version 4.1.1 and concentrations were calculated based on a standard curve derived from a recombinant cytokine standard. If the cytokine level in the sample was higher than the highest value on the standard curve, which occurred in many of the LPS stimulations, the highest value of the standard curve was reported for that data point. All samples were stimulated in parallel with ES

products diluted at 1:10 and 1:50, but only results from the ES concentration which generated optimal stimulation were reported. VEGF-C and VEGF-D production were analyzed by the Quantikine Immunoassay kits (R&D) as directed by the manufacturer.

5.3.6 Flow Cytometry of PBMCs and CD14+ Cells for Lymphatic-specific Surface Molecules

Cells were plated at $1x10^6$ PBMCs or $5x10^5$ CD14+ cells in 500 μL RPMI 1640 media supplemented with 10% FBS, 2 mM L-glutamine and antibiotics and stimulated with or without 100ng/mL LPS or ES (diluted at 1:10 and 1:50) for 72 hrs. Cells were stained for 30 min on ice in the dark according to manufacturers' direction using unconjugated rabbit anti-human VEGFR-3 (cat. 102-PA18AG; ReliaTech, Braunschweig, Germany), rabbit anti-human VEGR-2 (cat. 102-PA22AG; ReliaTech), mouse anti-human podoplanin (cat. 101-M40; ReliaTech) and rabbit anti-human LYVE-1 (cat. 102-PA50S; ReliaTech) primary antibodies and followed by secondary staining using FITC-labeled goat anti-rabbit (cat. 554020; BD Pharmingen) and PE-labeled goat anti-mouse (cat. 550589; BD Pharmingen). Cell events were acquired on a BD FACScan flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo (Tree Star, Ashland, Oregon). Cells stained with only the secondary antibody determined the thresholds for non-specific staining.

For the expression of VEGFR-3 on CD14+ cells isolated by MACS technology, cells were stained with primary antibodies rabbit anti-human VEGFR-3 (ReliaTech) and mouse anti-human CD14+ PE (BD Pharmingen) and followed by secondary staining with FITC-labeled goat anti-rabbit (BD Pharmingen). For the analysis we gated on the VEGFR-3+ cells in the $CD14+^{hi}$ population.

5.3.7 *In vitro* Matrigel Tubule Formation by LEC

LEC were released from the flask by gentle trypsinization (Lonza), washed, counted and $1x10^5$ LEC were stimulated in 200 µL EGM-2 MV SingleQuot media devoid of VEGF and spiked with 10 ng/mL IL-6 (R&D, Minneapolis, MN), 10 ng/mL IL-8 (Sigma, St. Louis, MO) or 1 ng/mL VEGF-A (R&D) for 10 min at 37 C before seeding. Cells were plated onto 100 µL Growth Factor-reduced Matrigel Matrix (BD Biosciences, Bedford, MA) which was used to coat a 24 well plate using the thin gel method in the manufacturer's instructions. After 24 hours, 5 randomized fields per well were photographed at 5x magnification on a Zeiss AxioVert 200M microscope (Carl Zeiss, Thornwood, NY). The images were opened and analyzed in AxioVision release 4.7.2. At a scaling ratio of 1:1 image analysis was performed; the total number of tubules was counted and the length of each tubule measured. Experiments were carried out in triplicate and representative data from one of four experiments is presented.

5.3.8 *In vivo* Matrigel Tubule Formation Assay

Rat carrier-free recombinant proteins including IL-8 (cat. 515-CN/CF), IL-6 (506-RL/CF), VEGF164 (564-RV/CF) were purchased from R&D Systems (Minneapolis, MI). Growth factor reduced Matrigel (cat 354230; BD Biosciences) was injected into August rats with or without 10ng/mL IL-8, 10ng/mL IL-6 or 10ng/mL VEGF-A as directed by the manufacturer. For the injections of the recombinant proteins, 3024 µL of liquid Matrigel was mixed with 576 µL of the recombinant rat lymphangiogenic proteins yielding a final concentration of 10 ng/mL for each protein; therefore, each animal received 80 µL of the recombinant rat lymphangiogenic factor in a 0.5 mL injection. In addition, we collected supernatants from PBMCs stimulated with or without worm ES

products (1:10) as previously mentioned. Supernatants from 5 different individuals were pooled and 576 μL of the pooled supernatants was mixed with 3024 μL of liquid Matrigel and 0.5 mL of this mix was injected into each rat (Carmi et al., 2009). These supernatants were analyzed by luminex bead technology using the Bio-Plex 8-plex kit (IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFNγ, TNFα) as well as IL-5, IL-13 and VEGF (Bio-Rad) according to the manufacturer's instructions as described above. There were 6 rats per group and each rat was injected s.c. into the dorsal intrascapular space of the animal. Regardless of Matrigel dilution with either recombinant proteins or PBMC supernatants, the Matrigel concentration was kept constant across all parameters and animals at 6.64 mg/mL. The Matrigel plugs were excised on day 10.

5.3.9 Immunohistochemistry

Specimens were processed, embedded in paraffin and sectioned on a rotary microtome at 4-5 μm. Sections were placed on slides coated with 2% 3-aminopropyltriethoxysilane and dried at 56 C overnight. The slides were subsequently deparaffinized in xylene and hydrated through descending grades of ethyl alcohol to distilled water. Slides were placed in Tris-buffered saline (TBS) pH 7.4 (Scytek Labs, Logan, UT) for 5 minutes for pH adjustment. Following TBS, podoplanin and IgG control slides underwent heat-induced epitope retrieval utilizing citrate buffer pH 6.0 (Scytek) in a vegetable steamer for 30 minutes at 100°C, allowed to cool on the counter at RT for 10 min and rinsed in several changes of distilled water. Von Willebrand (VWFactor) VIII slides underwent enzyme-induced epitope retrieval utilizing 0.03% Pronase E in TBS for 10 min at 37 C followed by running tap and distilled water rinses. Endogenous peroxidase was blocked utilizing 3% hydrogen peroxide and placed in a

methanol bath for 30 min followed by running tap and distilled water rinses. Following pretreatment, standard avidin-biotin complex staining steps were performed at RT on the Dako Autostainer (Dako North America, Inc., Carpinteria, CA). All staining steps were followed by two rinses in TBS + Tween 20 (Scytek). After blocking for non-specific protein respectively with normal horse serum or normal goat serum (Vector Labs, Burlingame, CA) for 30 minutes, sections were incubated with Avidin/Biotin blocking system for 15 minutes each (Avidin D, Vector Labs/d-Biotin, St. Louis, MO). Primary antibody slides were incubated for 60 min with the monoclonal mouse anti-rat podoplanin (cat. 104-M40; Relia Tech) diluted 1:400, the polyclonal rabbit anti-VWFactor VIII (cat. A0082; Dako) diluted 1:400, or the biotin-conjugated polyclonal rabbit anti-rat IgG (Novus Biologicals, Littleton, CO) diluted 1:100 in normal antibody diluent (NAD) (Scytek). Biotinylated horse anti-mouse rat absorbed IgG (H + L) (Vector) prepared at 10.0 μg/mL in NAD or goat anti-rabbit IgG (H+L) (Vector) at 11.0 μg/mL in NAD incubated for 30 min, followed by R.T.U. Vectastain Elite ABC Reagent incubation for 30 min (Vector). Reaction development utilized Vector Nova Red peroxidase chromogen incubation of 15 minutes and counterstain in Gill 2 Hematoxylin (Thermo Fisher, Kalamazoo, MI) for 15 seconds (podoplanin and VWFactor VII) or 90 seconds (rat IgG control), differentiation, and dehydration, clearing and mounting with synthetic mounting media. In addition, all slides underwent hematoxylin and eosin staining.

5.3.10 Statistics

The Signed Rank Test was used in the Statistical Analysis Software (SAS) version 9.1 to compare median cytokine and growth factor production by PBMCs in stimulated and control supernatants. The Signed Rank Test was also used to compare the

production of these factors by CD14+ monocytes compared to non-CD14 cells. GraphPad Prism 5 software (San Diego, CA) was used to carry out additional statistical analyses to compare the number of tubules per microscopic field in response to stimuli.

5.4 Results

5.4.1 Filarial ES Products Induce the Production of Lymphangiogenic Factors in PBMCs

We evaluated the ability of *Brugia* ES products to induce the secretion of molecules shown to exhibit lymphangiogenic potential in other systems. Human PBMCs were isolated from healthy volunteers and cultured with or without filarial ES products for 72 hours. The supernatant fluids were collected and analyzed for the production of the potentially lymphangiogenic molecules IL-3, IL-6, IL-7, IL-8 and VEGF-A by luminex technology. Cells cultured with filarial ES products secreted significantly higher levels of IL-8 ($p<0.001$), IL-6 ($p<0.004$) and VEGF-A ($p<0.002$) compared to cells cultured in media alone (Fig. 5.1). We did not detect IL-3 or IL-7 in any of our supernatants. We also attempted to measure VEGF-C and VEGF-D by ELISA, but these were below the limit of detection. These data suggest that *Brugia* ES products are capable of inducing the secretion of lymphangiogenic molecules by circulating PBMCs.

5.4.2 CD14+ Monocytes Are the Primary Producers of the Lymphangiogenic Molecules

Monocytes/macrophages have been shown to play an important role in the production of VEGFs in tumors and inflammation, so we hypothesized monocytes could be the PBMC in the periphery contributing to the production of IL-8, IL-6 and VEGF-A seen in response to worm ES products. We carried out CD14 fractionation experiments

using CD14 MACS technology to isolate CD14+ monocytes from total PBMCs. As seen in Fig. 5.2A and 5.2B CD14+ monocytes secreted significantly higher amounts of IL-8 ($p<0.0005$) and VEGF-A ($p<0.02$) compared to CD14-depleted cells in response to filarial ES products. However, CD14-enriched and depleted cell populations produced similar levels of IL-6 (Fig. 5.2B). CD14+ monocytes produced significantly more IL-8 ($p<0.02$) and VEGF-A ($p<0.02$) spontaneously compared to CD14-depleted cells (Fig 5.2A and 5.2C). CD14+ monocytes stimulated with *Brugia* ES products also secreted significantly higher levels of IL-8 ($p<0.02$) and IL-6 ($p<0.04$) compared to CD14+ cells cultured in media alone. LPS was used as a positive control for the production of IL-8 and IL-6; robust IL-8 and IL-6 responses were seen following LPS-stimulation compared to the lower responses to worm ES products, arguing that endotoxin contamination of ES batches is below levels associated with a detectable response. Taken together, these data suggest that CD14+ monocytes are the primary producers of the lymphangiogenic molecules IL-8 and VEGF-A in response to worm ES products, but CD14+ monocytes are not the major cell type contributing to the production of IL-6 in response to worm ES products.

5.4.3 Lymphatic-specific Surface Molecules on Circulating PBMCs and CD14+ Monocytes

In addition to the production of lymphangiogenic molecules, circulating cells have also been shown to support the development of lymphatic vasculature by differentiating into LECs; therefore, we examined the ability of *Brugia* ES products to promote the expression of lymphatic-specific surface molecules on PBMCs. As seen in Fig. 5.3 PBMCs stimulated for 72 hours with filarial ES products did not alter the

expression of VEGRF-3, LYVE-1, podoplanin or VEGFR-2. Additionally, LPS was not able to up-regulate these lymphatic-specific molecules on the surface of PBMCs. We were concerned the effects of the worm ES products might be specific for monocytes and would be masked in analyses of total PBMCs, so we isolated and stimulated CD14+ monocytes with *Brugia* ES products and examined the expression of VEGFR-3 on monocytes alone after 72 hours of incubation. Filarial ES products did not alter the expression of VEGFR-3 on CD14+ monocytes (Fig. 5.4). LPS-stimulation appeared to increase the expression of VEGFR-3 on monocytes but this finding was not statistically significant (Fig 5.4). Altogether, these findings suggest that exposure to worm ES products does not induce the expression of lymphatic markers or the differentiation of circulating cells to a lymphatic-like phenotype.

5.4.4 ES-induced Lymphangiogenic Mediators Stimulate LECs to Form Tubules *in vitro*

Since we were able to demonstrate the production of lymphangiogenic molecules by PBMCs in response to *Brugia* ES products, we examined the ability of these mediators detected following ES stimulation to alter LEC function as measured by tubule formation. LECs were layered on Matrigel cultures and stimulated with concentrations of IL-8, IL-6 and VEGF-A comparable to the amounts detected in supernatants of ES-stimulated PBMCs. After 24 hours, LECs cultured in the presence of IL-8, IL-6 and VEGF-A formed a more elaborate tubule network compared to cells cultured in media alone (Fig. 5.5A). Using image analysis software used to quantify tubule formation, cells cultured in the presence of IL-8, IL-6 or VEGF-A formed a greater number of tubules per microscopic field compared to LECs cultured without stimulus (Fig. 5.5B).

5.4.5 *In vivo* Vascularization of Matrigel Plugs by ES-induced Lymphangiogenic Molecules

Once we confirmed that the mediators we detected in ES-induced supernatants altered lymphatic function using an *in vitro* tubule formation assay, we wanted to establish the effects *in vivo*. To do so we injected rats with Matrigel containing IL-8, IL-6 or VEGF-A. We also collected PBMC-supernatants from PBMCs of 5 different individuals that were stimulated with ES products or cultured in media alone; these supernatant fluids were then pooled and tested for their ability to induce vessel formation *in vivo*. Characterization of the pooled PBMC supernatants which included measurable concentrations of IL-2, IL-6, IL-8 and VEGF is seen in Table 5.1. After 10 days, the plugs were excised and subjected to gross inspection to immediately identify vessel infiltration within the plugs (Fig. 5.6A and 5.6B). The plugs were then sectioned and hematoxylin and eosin (H&E) staining was carried out to examine cellular infiltration into the plugs. Compared to Matrigel alone injections, H&E staining revealed a dramatic cellular infiltration in Matrigel plugs that had been supplemented with IL-6, VEGF-A and supernatants from PBMCs stimulated with filarial ES products and to a lesser extent in Matrigels containing IL-8 or supernatants from PBMCs cultured in media alone (Fig. 5.6B). The Matrigel plugs were then subjected to immunohistochemical staining for the presence of von Willebrand Factor VIII (vWF) and podoplanin to identify blood and lymphatic vessels, respectively. Figure 5.6C shows positive staining for both vWF as well as podoplanin in plugs containing VEGF-A or supernatants from PBMCs stimulated to filarial ES products, indicating the presence of both blood and lymphatic vessels deep in the internal regions of the Matrigel plug.

5.5 Discussion

Lymphangiectasia, or the dilation of LVs, and lymphangiogenesis are subclinical features of filarial infection. Lymphatic vessels containing adult worms from infected individuals are characterized as distended, dilated, tortuous and highly indented (Vickery et al., 1985, Case et al., 1991, Rao et al., 1996, Figueredo-Silva et al., 2002). In dilated lymphatics, flow is impaired leading to improper drainage of interstitial fluids. The progression of mild lymphangiectasia to clinical lymphedema may be due to the accumulation of lymphatic fluid in the tissues over time following damage to the LVs. Defining the molecular mechanisms responsible for the development of lymphangiectasia may identify pathways that can be targeted to prevent lymphatic dysfunction and progression to lymphedema.

The specific factors responsible for the development of lymphangiectasia remain undefined, but our data suggest that parasite products are important in this process. Previously, it has been reported that lymphangiectasia was greatest near the worm nest and the removal or killing of worms could reduce lymphatic dilation arguing the importance of the parasite in the altering lymphatic pathology (Vickery et al., 1983, 1991, Case et al., 1991, Shenoy et al., 2009). Moreover, lymphangiectasia can be seen along the length of the infected vessel arguing that a soluble product secreted by the worm is responsible for the lymphatic pathology. We focused on the ES products released by the adult worms. Initially, we attempted to characterize the direct effects of ES products on LECs, but no measurable events were detected in our system. Subsequently, we hypothesized that ES products activate the lymphatic endothelium indirectly through an accessory cell. We have demonstrated that *Brugia* ES products stimulate host cells to

produce lymphangiogenic mediator; and that in pure form the same mediators alter LEC phenotype. *Brugia* ES products induce host PBMCs to secrete lymphangiogenic molecules IL-8, IL-6 and VEGF-A, and that when we used IL-8, IL-6 and VEGF-A, we induced LEC tubule formation at concentrations equivalent to those produced by ES-stimulated PBMCs. The production of these molecules could contribute to the development of lymphangiectasia in filarial-infected individuals.

Other studies have supported the role of parasite molecules in lymphangiogenesis and lymphangiectasia. Bennuru et al. showed filarial products can directly stimulate LEC proliferation and alter LEC junction adherence pathways which could contribute to lymphatic dilation (2009); however, the authors suggested that the microfilarial stage is responsible for LEC proliferation and differentiation. The microfilarial stage may contribute to the development of lymphatic disease, but since the microfilariae circulate in the blood vessels, which are not dilated in filarial-infected individuals, it is unlikely that microfilariae are the primary candidates responsible for the lymphangiectasia. Upon release from adult females, the microfilariae are found in the LVs for only a brief period of time before being removed by lymphatic flow, so it is not obvious that they are present in the LVs long enough to induce lymphatic dilation. Future studies to address the role of microfilariae will be required to define their role in altering lymphatic architecture.

Even though the expression of lymphangiogenic mediators is generally perceived to be beneficial for the formation of new LVs and to alleviate malfunctioning LVs (Karkkainen et al., 2001, Szuba et al., 2002, Yoon et al., 2003), the over-expression of lymphangiogenic molecules over an extended period of time has been shown to be detrimental and to impair lymphatic function. A massive expansion of the lymphatic

network can lead to defective LVs and thus decreased drainage and lymphedema. For example, VEGF-A and VEGF-C over-expression results in structurally and functionally abnormal and dilated lymphatics (Jeltsch et al., 1997, Nagy et al., 2002, Angeli et al., 2006).

Extending this argument, ES-induced host cells such as those found in PBMC preparations may compromise lymphatic function by secreting lymphangiogenic factors over many years throughout the duration of worm infection. The cumulative amounts/effects of these soluble mediators may parallel the over-expression model systems and lead to defective lymphatics. For instance, elevated plasma levels of lymphangiogenic factors such as VEGF-C have been found in microfilaremic individuals compared to endemic normal individuals (Debrah et al., 2006) suggesting the same VEGF and cytokine molecules involved in lymphangiogenesis and lymphangiectasia in other models are also present in filarial infection. Here, we detected the production of the lymphangiogenic molecules IL-8, IL-6 and VEGF-A in response to worm products. Given that adult worms can establish chronic infections and live for five to ten years, the production of these lymphangiogenic molecules over time in response to worm products may result in lymphatic damage as in other experimental model systems. Worms may initially induce the production of lymphangiogenic molecules to support their biological niche, but the continued production of these lymphangiogenic molecules over years of infection may result in dysfunctional LVs.

Even though we did see the production of VEGF-A by PBMCs in response to worm ES, we did not see the production of VEGF-C that was previously shown to be elevated in filarial-infected individuals (Debrah et al., 2006, Attout et al., 2009). We also

did not detect elevated levels of VEGF-D or lymphangiogenic cytokines IL-3 or IL-7. The lack of detection of VEGF-C, VEGF-D, IL-3 or IL-7 may be because we were examining the production of these molecules by PBMCs which may be the wrong cell source and these molecules may be produced by a cell found focally at the infection site. VEGF-C and VEGF-D signaling through VEGFR-3 is the primary and most well-characterized mechanism contributing to lymphangiogenesis, but there is an emerging role for VEGF-A in lymphangiogenesis (Nagy et al., 2002, Hong et al., 2004, Hirakawa et al., 2005, Wuest and Carr, 2010), so it is possible that this molecule may be playing an important role in filarial-induced lymphatic pathologies.

Monocytes and macrophages have been shown to play two major roles in supporting lymphangiogenesis. They can produce lymphangiogenic factors such as VEGFs and cytokines which induce LEC proliferation, survival, migration and tubule formation (Cursiefen et al., 2004, Maruyama et al., 2005). In this study we found that monocytes were primarily responsible for the production of IL-8 and VEGF-A in response to *Brugia* ES products; however we did not identify the cell type responsible for the production of IL-6, so future experiments need to be carried out to identify the source of IL-6 by PBMCs seen in response to worm ES products. In an alternative role, monocytes and macrophages can also transdifferentiate into LECs and incorporate into existing LVs (Maruyama et al., 2005). To address this alternative role of monocytes/macrophages in lymphangiectasia and lymphangiogenesis, we examined the expression of lymphatic-specific molecules on PBMCs in response to *Brugia* ES products, but we did not detect an up-regulation of any of these molecules. We carried out additional experiments looking at the expression of the lymphatic-specific molecule

VEGFR-3 on CD14+ monocytes isolated from PBMCs, but VEGFR-3 was not up-regulated on CD14+ cells. Taken together, these data suggest circulating cells do not adopt a LEC-like phenotype upon exposure to filarial ES products in our system. This could be a limitation of our use of only 72 hours of *in vitro* culture compared to years of *in vivo* worm exposure. Future experiments will be necessary to examine the expression of lymphatic-specific molecules on circulating cells from filarial-infected individuals.

Monocytes and macrophages may play a role in the lymphatic pathology associated with filarial infection. Typically, lymphatic vessels from infected individuals are thought to be devoid of an inflammatory response (Figueredo-Silva et al., 2002); however, some have noted small lymph thrombi composed of small mononuclear cells and multinucleated giant cells within the lumen (Vickery et al., 1985). Here, we defined CD14+ cells as the primary producer of IL-8 and VEGF-A in response to *Brugia* ES products but others have also reported the presence of monocytes/macrophages in regions of lymphangiectasia and lymphangiogenesis in infection with the filarid, *O. volvulus*. (Attout et al., 2009, Mackenzie et al., 2010). In nodules isolated from humans infected with *O. volvulus*, the predominant cell type associated with the worms was the macrophage and many of these cells stained positive for the lymphatic-specific marker LYVE-1 (Mackenzie et al., 2010). Attout et al. elegantly demonstrated macrophages from these nodules were positive for LYVE-1 and some were integrating into the lymphatic endothelium (2009). Taken together, these data suggest that monocytes/macrophages are important in lymphangiectasia and lymphangiogenesis in filarial infections and future research is needed to define the role of these cells in LF.

One could speculate the worm induces lymphangiogenesis and lymphangiectasia for many reasons. The worm may increase the vessel diameter for space; increasing the diameter of the vessel also slows lymphatic flow and increases the availability of nutrients and resources. The worm may stimulate expansion of the lymphatic network by inducing host production of VEGFs and cytokines to increase LEC proliferation and differentiation as a mechanism of LV dilation. We also demonstrated tubule formation in response to ES-stimulated mediators. Filarial worms may induce the formation of new lymphatic vessels to expand their biological niche for space and resources as well as an effort to maintain lymphatic flow through a collateral network. With the increased numbers of LVs, worms may also be guaranteeing their microfilariae reach the periphery to continue transmission and providing a transport network for many of the immunomodulatory molecules found within the ES.

In this study we have begun to dissect out the molecular mechanisms involved in the development of lymphangiectasia and lymphangiogenesis; however, similar studies must be carried out in cells isolated from endemic populations to confirm that the same the same molecules and cell types occur in filarial-infected individuals. Given that parasite products can induce the production of lymphangiogenic molecules by host cells and that infected persons exhibit lymphangiectasia, we hypothesize that these molecules are elevated in infected individuals. We are currently examining the production of VEGFs and cytokines by microfilaremic individuals and endemic normals as well as those with lymphedema in response to *Brugia* ES products. Since infected individuals exhibit lymphangiectasia, which may progress to a disabling lymphedema, we need to define the initial molecular mechanisms responsible for the development of disease.

Understanding the pathogenesis of LF may identify potential molecular targets for preventing disease progression as well as a greater understanding of the molecular mechanisms associated with lymphatic pathologies from cancer and inflammation.

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5.7 Figures and Tables

Figure 5.1. *Brugia* ES products induce the production of lymphangiogenic molecules by human PBMCs. PBMCs were isolated from healthy human volunteers and $1x10^6$ cells were seeded into tubes and stimulated with or without ES for 72 hrs. Cell supernatants were assessed for the presence of IL-8, IL-6 and VEGF-A by luminex bead analysis. *Brugia* ES products induced the production of (A) IL-8 (n=15), (B) IL-6 (n=10) and (C) VEGF-A (n=15) by PBMCs compared to cells in media alone as assessed by the Signed Rank test. Medians are presented as bars.

Figure 5.1

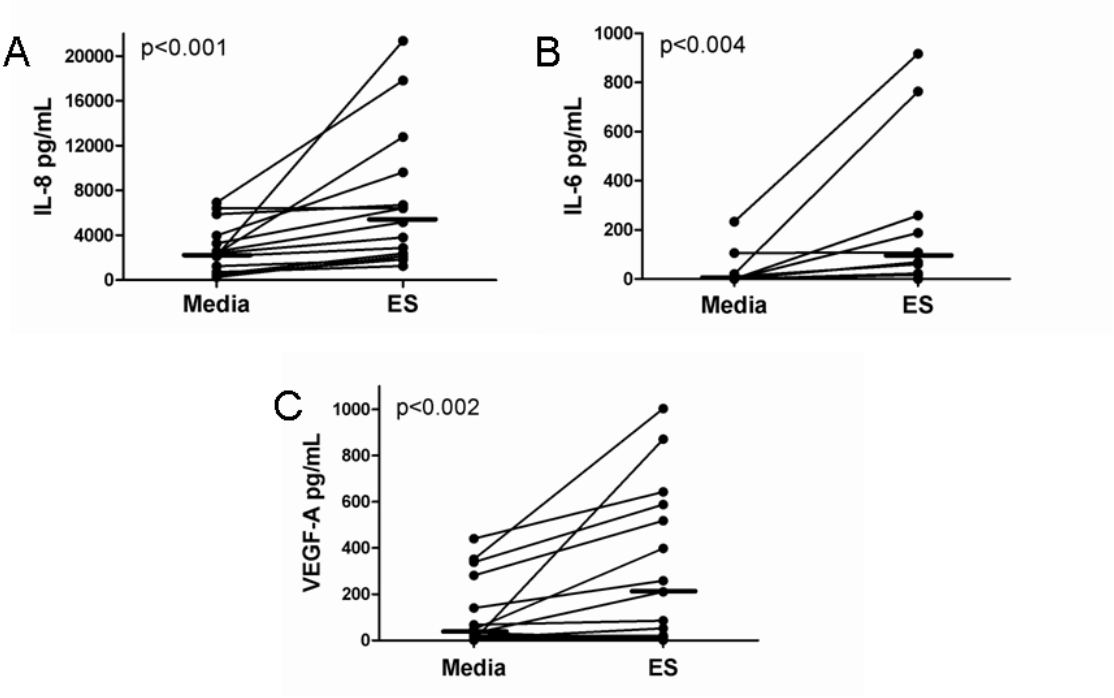

A
p<0.001
IL-8 pg/mL
20000
16000
12000
8000
4000
0
Media
ES
B
p<0.004
IL-6 pg/mL
1000
800
600
400
200
0
Media
ES
C
p<0.002
VEGF-A pg/mL
1000
800
600
400
200
0
Media
ES

Figure 5.2. *Brugia* ES products induce the production of IL-8 and VEGF-A by human CD14+ monocytes. In order to define the circulating cell type responsible for production of lymphangiogenic molecules by PBMCs in response to *Brugia* ES, human CD14+ monocytes were isolated by magnetic bead separation and compared to CD14-depleted cells for IL-8, IL-6 and VEGF-A production in response to worm ES products or LPS. Cell supernatants were assessed for the presence of (A) IL-8 (n=12), (B) IL-6 (n=7) and (C) VEGF-A (n=7) by luminex bead analysis after 72 hours of stimulation. Data presented represents the mean ± standard error of the mean (SEM) and comparisons were made using the Signed Rank test. LPS was used as a positive control and stimulated the production of IL-8 ($p<0.003$) and IL-6 ($p<0.02$) compared to cells cultured in media alone.

Figure 5.2

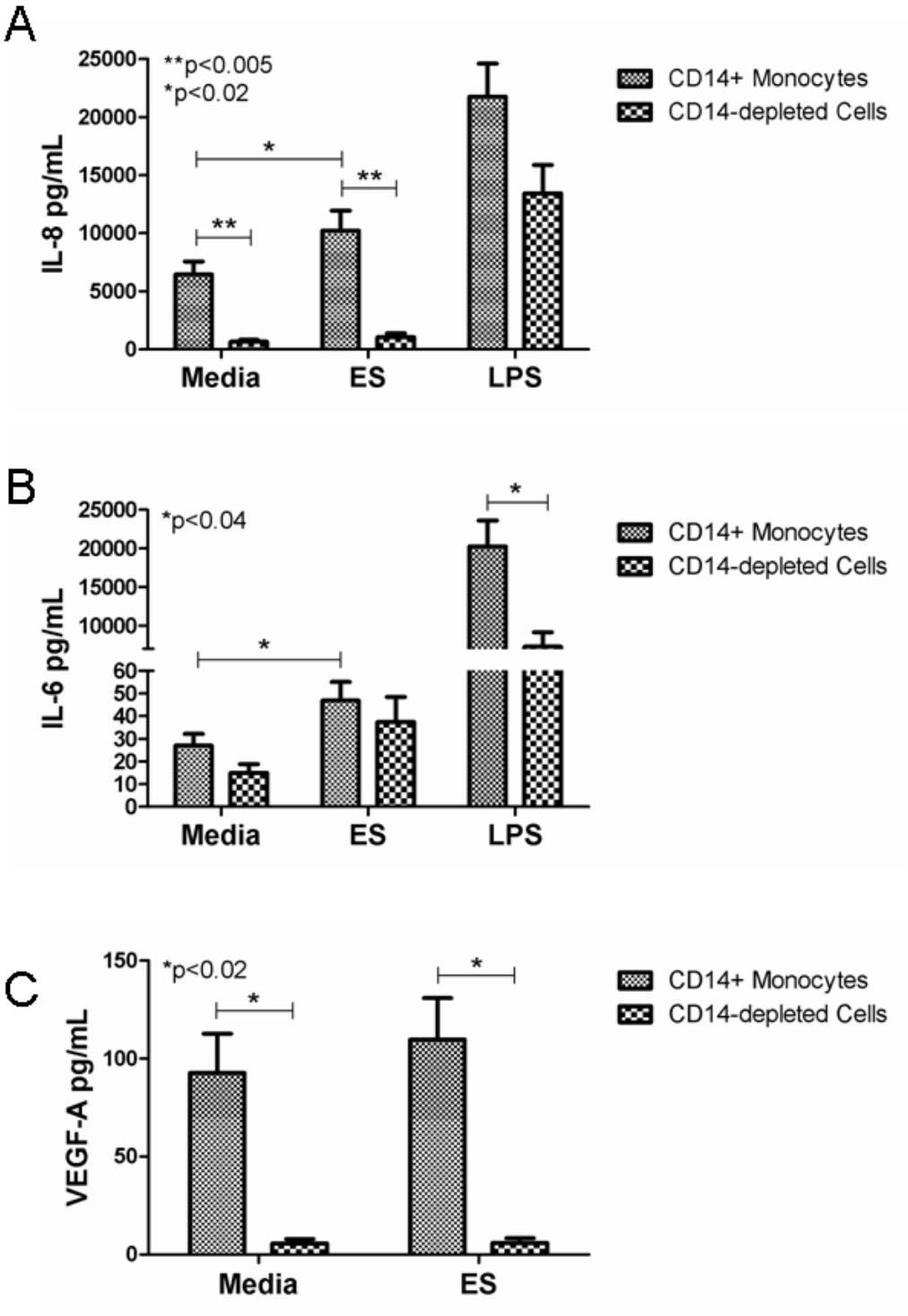

A
**p<0.005
*p<0.02
IL-8 pg/mL
25000
20000
15000
10000
5000
0
Media
ES
LPS
CD14+ Monocytes
CD14-depleted Cells
B
*p<0.04
IL-6 pg/mL
60
50
40
30
20
10
C
*p<0.02
VEGF-A pg/mL
150
100
50

Figure 5.3. Expression of lymphatic-specific markers on human PBMCs. Since PBMCs have been reported to transdifferentiate into a lymphatic-like phenotype, we measured the expression of lymphatic-specific surface molecules by flow cytometry on PBMCs (n>3) stimulated with *Brugia* ES products or LPS for 72 hours. Medians are presented as bars. Lymphatic-specific markers were not up-regulated following stimulation with ES products or LPS, compared to media alone.

Figure 5.3

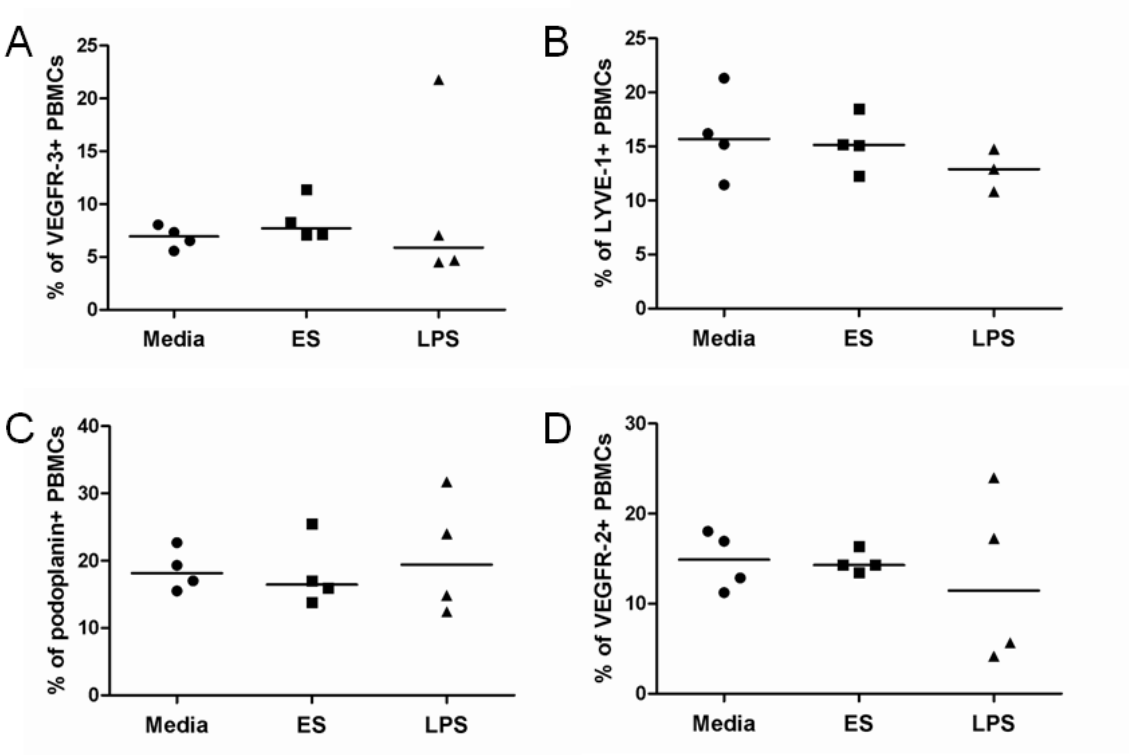

A
% of VEGFR-3+ PBMCs
25
20
15
10
5
0
Media
ES
LPS
B
% of LYVE-1+ PBMCs
25
20
15
10
5
0
Media
ES
LPS
C
% of podoplanin+ PBMCs
40
30
20
10
0
Media
ES
LPS
D
% of VEGFR-2+ PBMCs
30
20
10
0
Media
ES
LPS

Figure 5.4. Expression of VEGFR-3 on CD14+ monocytes. Given the lack of changes in expression in lymphatic-specific molecules on the total PBMC population stimulated with worm ES products, we measured the expression of the lymphatic-specific molecule VEGFR-3 on CD14+ monocytes (n=17) by flow cytometry in response to *Brugia* ES products or LPS for 72 hours. Medians are presented as bars.

Figure 5.4

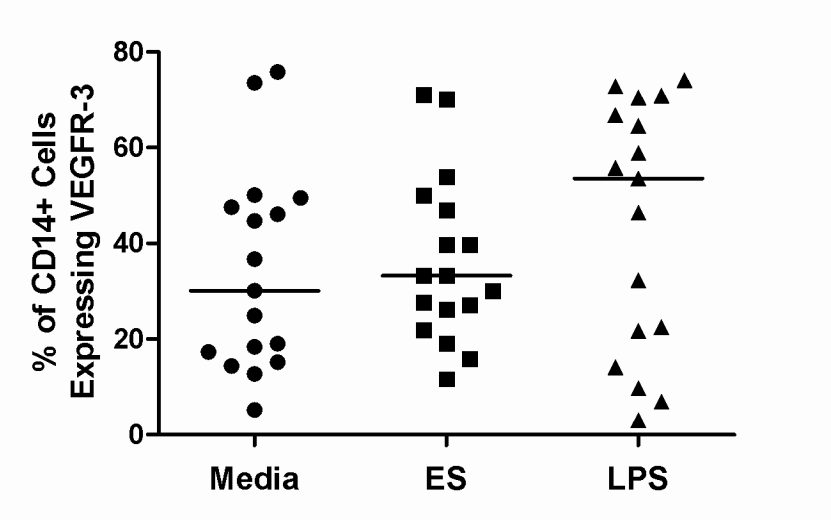


Figure 5.5. Filarial ES-induced lymphangiogenic mediators induce LEC tubule formation *in vitro*. Lymphangiogenic mediators found in ES-stimulated PBMC supernatants were tested for their ability to induce LEC tubule formation during *in vitro* Matrigel culture. LECs were grown on Matrigels in the presence or absence of IL-8, IL-6 or VEGF-A and lymphatic networks were photographed (A). (B) The number of tubules was quantified using image analysis software. The experiments (n=4) were performed in triplicate and the data presented are the medians of one representative experiment.

Figure 5.5

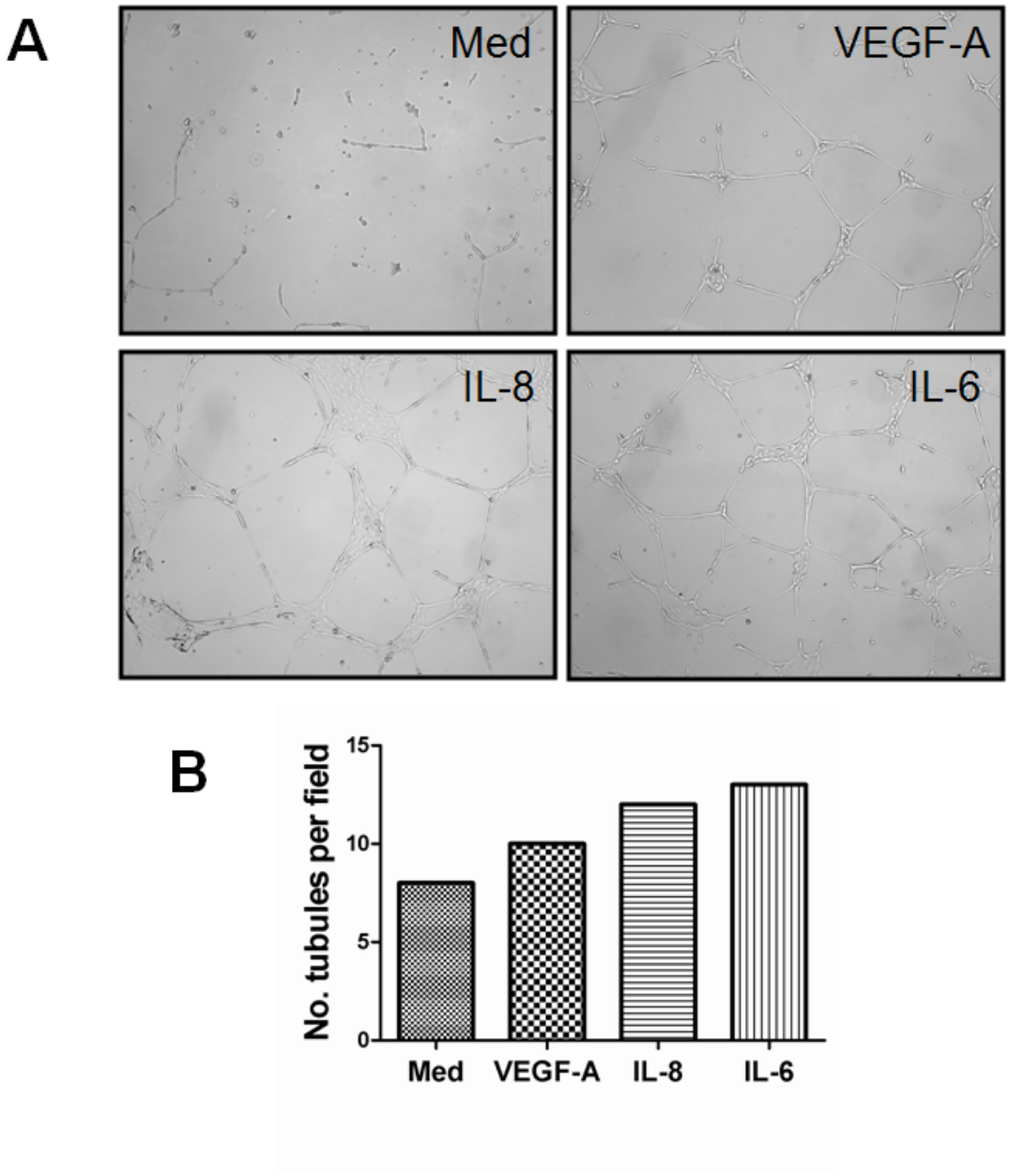

A
Med
VEGF-A
IL-8
IL-6
B
No. tubules per field
15
10
5
0
Med
VEGF-A
IL-8
IL-6

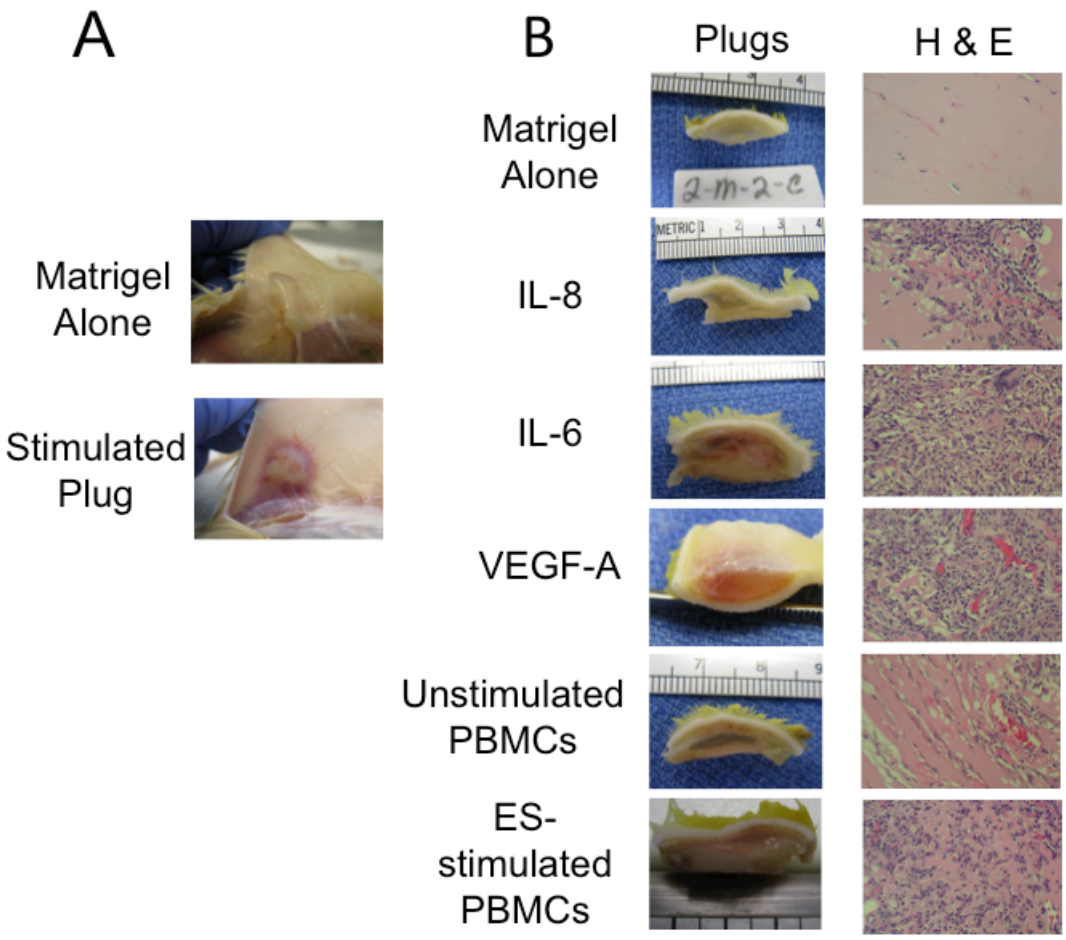
Table 5.1.
Cytokine and growth factor levels (pg/mL) in PBMC supernatants[a]

	Unstimulated supernatants	ES-stimulated supernatants
IL-2	3.07	4.41
IL-4	Undetectable	Undetectable
IL-5	Undetectable	Undetectable
IL-6	18.58	68.8
IL-8	5061.76	30898.94
IL-10	Undetectable	Undetectable
IL-13	Undetectable	Undetectable
GM-CSF	Undetectable	Undetectable
IFNγ	Undetectable	Undetectable
TNFα	Undetectable	Undetectable
VEGF	46.04	115.65

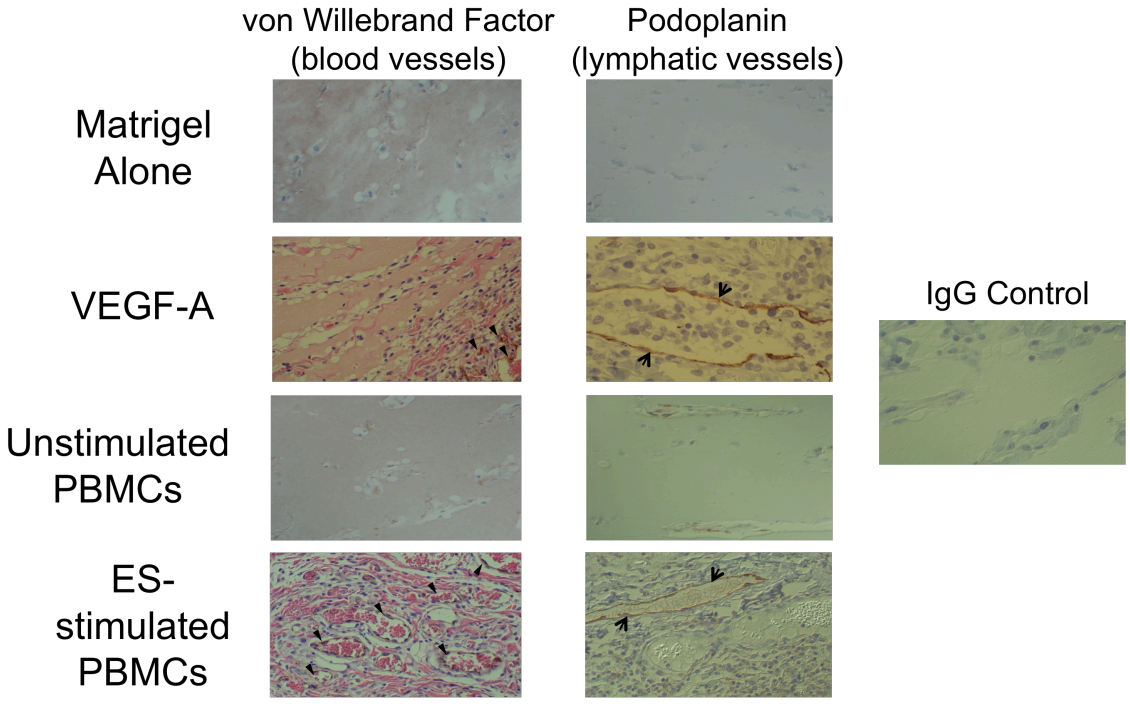
[a] $1x10^6$ PBMCs were stimulated with worm ES or cultured in media alone for 72 hours. Supernatants from 5 different individuals were pooled and cytokines and growth factors were analyzed by luminex bead technology. Matrigel plugs were supplemented with 80 μL of the pooled supernatants and used for rat *in vivo* vessel formation experiments.

Figure 5.6. Filarial ES-induced lymphangiogenic mediators stimulate vessel formation in rats. Matrigel with or without IL-8, IL-6, VEGF-A, supernatants collected from PBMCs stimulated with filarial ES products or supernatants collected from PBMCs cultured in media alone, was injected into August rats (6 rats per stimulus) and after 10 days Matrigel plugs were excised. (A) A representative example of a plug that had not been vascularized compared to a plug that had been vascularized upon initial examination of the plug in the subcutaneous layer. (B) Each plug was excised and sectioned for H&E staining and representative plugs from each stimulus after excision are shown. Representative H&E images for each stimulus are also seen here demonstrating a dramatic cellular infiltrate in the stimulated Matrigel plugs compared to Matrigel alone. (C) Immunohistochemical analysis on sections from Matrigel plugs that demonstrated a cellular infiltration based on their H&E staining including Matrigel plugs supplemented with VEGF-A or supernatants from PBMCs stimulated with worm ES products. Matrigel sections were examined for the presence of blood and lymphatic vessels based on their staining of von Willebrand Factor VIII or podoplanin, respectively. Sections were also stained with anti-rat IgG as a staining control.

Figure 5.6


A
Matrigel Alone
Stimulated Plug
B
Plugs
H & E
Matrigel Alone
IL-8
IL-6
VEGF-A
Unstimulated PBMCs
ES-stimulated PBMCs

C


von Willebrand Factor
(blood vessels)
Podoplanin
(lymphatic vessels)
Matrigel
Alone
VEGF-A
IgG Control
Unstimulated
PBMCs
ES-
stimulated
PBMCs

CHAPTER 6

EVALUATING LYMPHANGIOGENIC FACTORS AND CELLS IN FILARIAL-ENDEMIC POPULATIONS[1]

[1]Weinkopff, T., and P. Lammie. 2010. To be submitted to *PLoS NTDs*.

6.1 Abstract

Lymphatic filariasis (LF) is caused by the parasitic nematodes *Wuchereria bancrofti* and *Brugia malayi* which infect over 120 million people worldwide. Most microfilaria-positive (Mf+) individuals appear asymptomatic, but exhibit subclinical manifestations such as lymphangiectasia or the dilation of lymphatic vessels. We hypothesized that the excretory-secretory products (ES) of the worms activate the lymphatic endothelium and have demonstrated a role for monocyte-derived lymphangiogenic mediators produced by healthy naïve volunteers in response to filarial ES products in altering lymphatic endothelial cell differentiation. However, it is unclear if the same lymphangiogenic mediators are produced by individuals living in endemic regions of LF. Therefore, we evaluated the production of IL-8, IL-6 and VEGF-A by PBMCs from Mf+ persons as well those with clinical lymphedema (Le) and endemic normal (EN) controls. All groups (Mf+, EN, Le) produced significant levels of IL-8 and IL-6 in response to filarial ES products and Mf+ persons produced higher levels of antigen-induced VEGF-A compared to ENs. Mf+ individuals also exhibited a higher frequency of CD14+ monocytes producing IL-8 and IL-6 than EN. Phenotypic analysis of these cells CD14+ IL-8+ and CD14+ IL-6+ cells revealed concurrent expression of CD68, CD163, CD206, HLA-DR and TNFα that suggesting these cells have characteristics of both alternatively and classically activated macrophages. Collectively, these data suggest that filarial-endemic populations do produce lymphangiogenic factors in response to worm ES products and that monocytes and their secreted products may be contributing to the lymphangiectasia seen *in vivo* and thus the development of lymphedema.

6.2 Introduction

Lymphatic filariasis (LF) is caused by the parasitic nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* which infect over 120 million people worldwide and cause severe morbidity in an additional 40 million individuals (Ottesen et al., 1997, 2008). LF is characterized by a wide spectrum of disease manifestations including asymptomatic microfilaremic individuals with active infection as well as individuals with the overt clinical manifestations of the disease like lymphedema and hydrocele (Figueredo-Silva et al., 2002). Even though microfilaremic individuals appear asymptomatic, they exhibit subclinical manifestations such as lymphangiectasia or the dilation of the lymphatic vessels (LV) (Amaral et al., 1994, Freedman et al., 1994, 1995, Noroes et al., 1996a, 1996b, Dreyer et al., 2000, 2002, Figueredo-Silva et al., 2002, Shenoy et al., 2008, 2009). For instance, ultrasonography revealed 50% of microfilaremic males exhibited dilated and tortuous lymphatics and all men with detectable worms have dilated lymphatics even though they appear asymptomatic (Amaral et al., 1994, Noroes et al., 1996b). Removal or killing of worms by drug treatment reverses the lymphatic dilation suggesting the parasite is responsible for the dilation seen *in vivo* (Vickery et al., 1983, 1991). In addition, lymphangiectasia is seen in SCID mice arguing that the adaptive immune response is not involved in the modulation of the lymphatic tissue (Vincent et al., 1984, Nelson et al., 1991). Furthermore, histopathologic examinations of LVs harboring living adult worms demonstrated the lack of an inflammatory response (Amaral et al., 1994, 2000, Figueredo-Silva et al., 2002).

Interestingly, the lymphatic dilation is not restricted to the site of the worm nest, but found throughout the length of the infected vessel, suggesting a soluble factor secreted by the parasite could be responsible for the dilation seen *in vivo* (Amaral et al., 1994, Dreyer et al., 2000, Figueredo-Silva et al., 2002).

The mechanisms responsible for the lymphatic dilation seen in infected vessels remain elusive, but this early lymphangiectasia may serve as the initiation event for the development of lymphedema. Therefore, we wanted to define the molecular mechanisms contributing to the lymphangiectasia and lymphangiogenesis associated with filarial infection. Previous findings from our lab have suggested that filarial ES products do not induce a direct activation of LECs (Chapter 3); however, worm ES products indirectly activate LECs through the production of lymphangiogenic molecules by human peripheral blood mononuclear cells (PBMC) from healthy naïve volunteers and monocytes are the primary cell source for these lymphangiogenic factors (Chapter 4). Lymphangiogenic molecules such as the cytokines IL-8, IL-6 and members of the vascular endothelial growth factor (VEGF) family are produced by human PBMCs in response to filarial ES products and are known to support LEC proliferation, survival, migration and tubule formation (Koch et al., 1992, Holzinger et al., 1993, Joukov et al., 1996, 1997, Lee et al., 1996, Achen et al., 1998, Makinen et al., 2001, Nagy et al., 2002, Podgrabinska et al., 2002, Li et al., 2003, Hirakawa et al., 2005, Yao et al., 2006, Adams and Alitalo, 2007, Wuest and Carr, 2010, Chapter 4).

Given our previous findings and studies by Debrah et al. suggesting that microfilaremic individuals exhibit higher levels of lymphangiogenic factors in their plasma compared to endemic normals, we wanted to determine if circulating cells from filarial-infected individuals also exhibited the ability to produce lymphangiogenic molecules in response to worm ES products (2006). Here, we show the production of lymphangiogenic molecules by PBMCs from microfilaremics as well as endemic normals and those with clinical lymphedema in response to filarial ES products. Microfilaremic individuals exhibit higher frequencies of IL-8- and IL-6-producing monocytes and phenotypic analysis of these cells demonstrates characteristics of both classically and alternatively activated macrophages.

6.3 Materials and Methods

6.3.1 Parasite Materials and Collection of ES Products

Brugia malayi adult female worms were collected from the peritoneal cavity of infected jirds, *Meriones unguiculatus*, that were obtained from the NIAID Filariasis Repository at the University of Georgia (Athens, GA). ES products were collected as previously reported (Chapter 4) and stored at 4 C until further use. Prior to cell stimulations with ES products, ES products were filtered using 0.45 μm Millex-HA syringe filters (Millipore, Carrigtwohill, Co. Cork, Ireland) and used in a dose-dependent (diluted at 1:10, 1:50) manner across various replicates and batches to maintain reproducibility. All batches of ES products were tested for endotoxin activity using the Limulus Amebocyte Lysate QCL-1000 assay (Lonza, Walkersville, MD) and ES products were only used at concentrations ≤ 0.1 eu/mL for cell stimulations.

6.3.2 Isolation of Peripheral Blood Mononuclear cells

Human PBMCs were collected from clinically- and parasitologically-characterized samples isolated from several groups of patients as part of previous IRB-approved studies in Haiti (Lammie et al., 1993, Dimock et al., 1994, 1996). PBMCs were collected from microfilariae-positive (Mf+) persons, those with lymphedema (Le), and residents who live in filarial-endemic areas of Haiti, but are not infected. The latter individuals are termed endemic normals (EN).

For isolation of the Haitian PBMCs, lymphocyte separation media (MP Biomedicals, Solon, OH) was used as directed by the manufacturer. In brief, blood was collected from donors by venipuncture in 10mL ethylenediaminetetraacetic acid (EDTA) Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), centrifuged for 10 min at 1000 x g at 4 C and the buffy coat was removed, resuspended in 2 mL RPMI 1640 media and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and layered over 3 mL lymphocyte separation media. Cells were centrifuged for 30 min at 1000 x g at 4 C, the buffy coat was removed, washed and cryopreserved.

6.3.3 Production of Lymphangiogenic Factors by PBMCs

Cells were plated at $1x10^6$ PBMCs in 500 µL RPMI 1640 media (GIBCO) supplemented with 10% FBS, 2 mM L-glutamine and antibiotics and cultured with or without 100ng/mL LPS or ES diluted at 1:10, 1:50 for 72hrs. Cell culture supernatants were collected and analyzed for IL-8, IL-6 and VEGF-A by luminex technology using the Bio-Plex Pro multiplex suspension array system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. In short, cell culture supernatant fluids were incubated with fluorescently-labeled coupled magnetic beads for 30 min at room temperature (RT)

on a shaker in a 96 well filter plate. Magnetic beads were washed 3 times and incubated with a multiplex mixture of the detection antibodies for 30 min at RT on a shaker. The beads were washed 3 times and incubated with streptavidin-PE for 10 min at RT on a shaker. The beads were washed 3 more times, resuspended in 125 µL of assay buffer, shaken and the plate was read using low PMT settings. Data were analyzed by the Bio-Plex Manager software version 4.1.1 and concentrations were calculated based on a standard curve derived from a recombinant cytokine standard. If the cytokine level in the sample was higher than the highest value on the standard curve, which occurred in many of the LPS stimulations, the highest value of the standard curve was reported for that data point. All samples were stimulated in parallel with ES products diluted at 1:10 and 1:50, but only the optimal ES stimulation was reported.

6.3.4 Flow Cytometry of PBMCs for Intracellular IL-6 and IL-8

Cells were plated at $5x10^5$ PBMCs in 200 µL RPMI 1640 media supplemented with 10% FBS, 2 mM L-glutamine and antibiotics and cultured with or without ES (1:10) for 22 hrs and 48 hrs for IL-8 and IL-6 production, respectively. During the last 4 hrs of stimulation, cells were cultured in the presence of GolgiStop (BD Biosciences, San Diego, CA). To compare the levels of intracellular IL-6 and IL-8 between PBMCs from Mf+ and EN persons, cells were stained for 30 min on ice in the dark according to manufacturer's direction with FITC-labeled mouse anti-human CD14 (cat. 555397; BD Pharmingen). After washing, cells were fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, San Jose, CA) according the manufacturer's instructions. Cells were then stained with PE-labeled rat anti-human IL-6 (cat. 559331; BD Pharmingen) or PE-labeled mouse anti-human IL-8

(cat. 554720; BD Pharmingen). For phenotypic characterization of CD14+ cells, cells were stained with PECy5-labeled mouse anti-human CD14 (cat. Ab25395; Abcam), FITC-labeled mouse anti-human CD68 (cat. 11068973; eBioscience), FITC-labeled mouse anti-human CD163 (cat. T1062; BMA Biomedicals), FITC-labeled mouse anti-human CD206 (cat. 551135; BD Pharmingen), FITC-labeled mouse anti-human HLA-DR (cat. 560944; BD Pharmingen) and/or FITC-labeled mouse anti-human TNFα (cat. 11-7349; eBioscience). PBMCs were then washed, fixed and permeabilized for intracellular staining for IL-6 and IL-8 as previously described. Cell events were acquired on a BD FACScan flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo (Tree Star, Ashland, Oregon). For the analysis we gated on the $CD14+^{hi}$ population since some individuals possessed a CD14 intermediate population.

6.3.5 Statistics

Statistical analyses were performed using GraphPad Prism 5 software (San Diego, CA). The Wilcoxon Signed Rank test was used to compare median cytokine and growth factor production by PBMCs in stimulated and control supernatants. The Mann Whitney test was to compare cytokine and growth factor production as well as the number of CD14+ IL-8+ and CD14+ IL-6+ cells between different groups (Mf+, EN, Le).

6.4 Results

6.4.1 Filarial ES Products Induce the Production of Lymphangiogenic Factors in PBMCs

In order to evaluate the response to ES products in human filarial infection, human PBMCs from patients living in a filarial-endemic region of Haiti were cultured with or without ES products for 72 hours. Cells were isolated from three different patient

groups including Mf+ individuals, those with clinical lymphedema (Le) and EN controls. Given the production of lymphangiogenic molecules IL-8, IL-6 and VEGF-A by naïve PBMCs from healthy volunteers in response to filarial ES products (Chapter 4), we examined the supernatant fluids of ES-stimulated Haitian PBMCs for the presence of IL-8, IL-6 and VEGF-A by luminex technology. Cells from ENs, Mf+ individuals and patients with lymphedema all produced significantly elevated levels of IL-8 in response to ES products (ENs $p<0.05$, Mf+ $p<0.05$, LE $p<0.05$) compared to unstimulated cells (Fig. 6.1A). Similarly, IL-6 production by PBMCs in response to filarial ES products was also elevated in each of the three groups (ENs $p<0.0005$, Mf+ $p<0.009$, LE $p<0.05$) compared to cells cultured in media alone (Fig. 6.1B). However, the same trend was not detected in cells stimulated with worm ES products for VEGF-A production, but PBMCs from Mf+ individuals did produce significantly greater levels of antigen-induced VEGF-A ($p<0.04$) compared to ENs (Fig. 6.1C). For all groups, LPS (10 ng/mL) was used as a positive control for the induction of IL-8, IL-6 and VEGF-A. Stimulation with LPS induced production of IL-8, IL-6 and VEGF-A at significantly increased amounts compared to cells cultured in media alone (Fig. 6.1A, 6.1B and 6.1C).

6.4.2 Frequencies of Monocytes Producing Lymphangiogenic Factors

Monocytes have been shown to play important roles in lymphangiogenesis in tumors, wound healing models and in other inflammatory settings. We have also shown CD14+ monocytes from naïve healthy volunteers are the primary producers of the lymphangiogenic molecules in response filarial ES products (Chapter 4). As a result, we compared the frequencies of CD14+ monocytes producing IL-8 and IL-6 between Mf+ and EN individuals. PBMCs were cultured with or without filarial ES products;

intracellular cytokine staining followed by flow cytometric analysis was carried out to identify CD14+ monocytes producing IL-8 and IL-6. PBMCs were cultured in the presence and absence of filarial ES products, but we did not see an increase in the number of cells double-positive for CD14 and IL-8 or CD14 and IL-6 in response to ES products (data not shown) so the data reported here display the frequency of CD14+ monocytes spontaneously producing each lymphangiogenic molecule. We detected a population of CD14+ IL-8+ cells at greater frequencies in Mf+ compared to EN individuals (Fig. 6.2A and 6.2B). Figure 6.2A shows representative flow cytometric data from one Mf+ individual and one EN, but if we compare groups, Mf+ persons have significantly elevated numbers of CD14+ IL-8+ cells ($p<0.04$) compared to ENs (Fig. 6.2B). Similar experiments were carried out for the detection of monocytes producing IL-6 and Figure 6.2C shows representative data for from one Mf+ and one EN person. Mf+ persons appear to have more PBMCs staining double-positive for CD14 and IL-6 compared to ENs, but this difference was not significant (Fig. 6.2D).

6.4.3 Phenotypic Characterization of CD14+ Monocytes Producing IL-8 and IL-6

The monocytes involved in vessel formation in tumor and wound healing models have been characterized as possessing anti-inflammatory and proangiogenic properties and have been termed M2 monocytes/macrophages (Mantovani et al., 2002, 2007). These M2 monocytes also exhibit an alternatively activated phenotype (Mantovani et al., 2002, 2007). Monocytes characterized by an alternatively activated phenotype have also been demonstrated in filarial infections in humans and animals (Loke et al., 2000, 2002, Nair et al., 2003, Babu et al., 2009). Therefore, we wanted to phenotypically characterize the CD14+ monocytes identified in Mf+ and ENs based on their expression of M2-

associated molecules such as CD163, a scavenger receptor, and CD206, a mannose receptor (Mantovani et al., 2002, Gordon, 2003). Following our gating strategy (Fig. 6.3A), CD14+ IL-8+ cells expressed both CD163 and CD206 (Fig. 6.3B) and neither CD163 nor CD206 was up-regulated in response to filarial ES products (6.3C and Table 6.1). In addition, there were no significant differences in CD163 or CD206 expression levels on CD14+ IL-8+ monocytes between Mf+ and EN persons (6.3B and Table 6.1). Using a similar gating strategy (Fig. 6.3D) for CD14+ IL-6+ monocytes, both CD163 and CD206 were expressed on these cells (Fig. 6.3E) but CD163 and CD206 were not up-regulated in response to worm ES products (Fig. 6.3F and Table 6.2); moreover, there were no differences in the expression levels of CD163 or CD206 on CD14+ IL-6+ monocytes between Mf+ and EN individuals (Fig. 6.3E and Table 6.2). We also evaluated the production of TNFα, a typical classically activated M1 molecule and found that the CD14+ IL-8+ and CD14+ IL-6+ monocytes from both Mf+ and ENs were producing TNFα (Fig. 6.3B and 6.3E).

Additionally, we also examined the maturation state of the IL-8- and IL-6-producing CD14+ from filarial-endemic populations based on their CD68 expression, a known macrophage maturation marker (Pulford et al., 1990). The CD14+ monocytes producing IL-8 expressed CD68 (Fig. 6.3B) confirming their monocye/macrophage lineage but they did not up-regulate CD68 in response to filarial ES products (Fig. 6.3C) and there were no differences in CD68 expression levels between groups (Fig. 6.3B and Table 6.1).

IL-6-producing CD14+ monocytes also expressed CD68 (Fig. 6.3E); stimulation with worm ES products did not up-regulate CD68 on IL-6-producing monocytes (Fig. 6.3F) and there was no difference in CD68 expression levels between Mf+ and ENs (Fig. 6.3E and Table 6.2).

CD14+ IL-8+ and CD14+ IL-6+ monocytes also expressed HLA-DR on their surface (Fig. 6.3B and 6.3E); however there were no significant differences in HLA-DR expression levels between groups (Table 6.1 and 6.2).

6.5 Discussion

Lymphangiectasia or the dilation of LVs is a hallmark of filarial infection. Microfilaremic individuals appear asymptomatic but they exhibit subclinical lymphangiectasia; in contrast, individuals with lymphedema are typically antigen-negative. It has been hypothesized that the parasite is responsible for modulating the LECs lining the infected vessels to induce lymphangiectasia and that the early dilation seen in filarial-infected individuals will lead to clinical lymphedema. However, the molecular mechanisms responsible for the LV dilation seen in LF remain undefined. Previous findings have not clarified the role of parasite molecules in the direct activation of LECs. For example, endothelial cell proliferation was not detected in HUVECs stimulated with worm extract *in vitro* (Rao et al., 1996); however, Bennuru and Nutman have shown LEC proliferation and differentiation as measured by tubule formation in response to *Brugia* parasite extract *in vitro* (2009).

These studies have provided a foundation for our understanding of filarial pathology, but they both utilized parasite extracts in their experimental design and LV dilation is associated with living adult worms and found along the length of the infected vessels; therefore, we examined the ability of ES products secreted by the adult worms to induce LEC activation.

Similarly to Rao et al., experiments in our lab have not revealed a direct activation event such as proliferation by LECs in response to filarial ES products, but we have detected the production of lymphangiogenic mediators by PBMCs from healthy naïve donors in response to worm ES products (1996, Chapter 3 and Chapter 4). Consequently, we hypothesized that the same lymphangiogenic molecules would be generated in response to worm ES products in filarial-infected individuals. Here, we demonstrate the production of IL-8 and IL-6 in response to worm ES products by PBMCs from all 3 groups including Mf+, Le and ENs (Fig. 6.1). This is consistent with previous findings where LEC proliferation was seen in response to serum from Mf+ persons suggesting host-derived factors can induce LEC proliferation which may provide the underlying mechanism for lymphangiectasia (Bennuru and Nutman 2009).

Our findings did not suggest differential production of lymphangiogenic mediators between groups; PBMCs from most individuals, irrespective of their infection or disease status, responded to stimulation with ES products. Even though all PBMCs produced lymphangiogenic molecules in response to worm ES products, modulation of the lymphatics will be restricted to the Mf+ patient group since they are harboring the adult worms and are being continuously exposed to ES products. The sustained production of lymphangiogenic mediators in response to parasite molecules over the

course of a filarial infection, sometimes years, may lead to impaired lymphatic function. Lymphangiogenic molecules and lymphangiogenesis have been considered beneficial for acute inflammatory responses, wound healing and for the improvement of lymphatic dysfunction associated with secondary lymphedema; but, lymphangiogenesis can also be detrimental, especially under chronic inflammatory conditions, and result in dysfunctional LVs (Jeltsch et al., 1997, Nagy et al., 2002, Yoon et al., 2003, Hong et al., 2004, Angeli and Randolph, 2006, Kataru et al., 2009). Further, over-expression of VEGF-A and VEGF-C results in structurally and functionally abnormal and enlarged lymphatics containing incompetent valves with a reduced flow and delayed lymph clearance (Jeltsch et al., 1997, Nagy et al., 2002, Angeli and Randolph, 2006). In patients with chronic inflammatory conditions such as psoriasis, rheumatoid arthritis and Crohn's disease, VEGF-A is up-regulated in inflamed tissue and associated with abnormally large lymphatics (Braverman and Yen, 1974, Detmar et al., 1994, Koch et al., 1994, Kanazawa et al., 2001, Xu et al., 2003, Pedica et al., 2008). In addition, tumors over-expressing VEGF-C induce the growth of immature and malfunctional lymphatics in the periphery (Isaka et al., 2004). Taken together, these studies suggest lymphangiogenesis is beneficial in many settings, but once the balance has been shifted and lymphangiogenic factors are elevated or present for prolonged periods of time, there is a negative impact on lymphatic function. Given the chronicity of filarial infection, the lymphatic dilation seen in LF may be a result of increased or prolonged production of lymphangiogenic molecules.

Monocytes/macrophages have been implicated in lymphangiogenesis and lymphangiectasia in a variety of inflammatory conditions such as tumors and wound healing (Kerjaschki, 2005, Maruyama et al., 2007, Xing et al., 2009). Monocytes/macrophages are important in inflammatory lymphangiogenesis because they produce lymphangiogenic mediators such as cytokines and VEGFs in response to stimuli, they are present within and around LVs of inflamed tissue and the depletion of macrophages by clondronate liposomes reduces lymphangiogenesis (Cursiefen et al., 2004, Kerjaschki, 2005, Maruyama et al., 2005, Kataru et al., 2009). Interestingly, monocytes/macrophages can also transdifferentiate into LECs themselves (Maruyama et al., 2005). Given their established role in lymphangiogenesis and previous findings in our lab suggesting monocytes are the primary producers of lymphangiogenic molecules in response to filarial ES products, we evaluated the presence of CD14+ monocytes producing IL-8 and IL-6 in filarial-infected individuals (Chapter 4). Microfilaremic individuals exhibited a higher frequency of CD14+ monocytes making IL-8 and IL-6 compared to ENs (Fig. 6.2). Elevated numbers of cells with lymphangiogenic potential in Mf+ persons may be contributing to the lymphangiectasia seen in this patient population.

We carried out a phenotypic characterization of the CD14+ monocytes producing IL-8 and IL-6 from filarial-infected individuals and found these cells expressed CD68, CD163 and CD206 (Fig. 6.3 and Fig. 6.4). Their CD68 expression in addition to their CD14 positivity confirms their monocyte/macrophage lineage. These cells also expressed HLA-DR suggesting they have the characteristics of antigen presenting cells. The cells were positive for CD163, a cysteine-rich group B scavenger receptor, and

CD206, a mannose receptor, which have been shown to be markers of M2-polarized monocytes/macrophages, also termed alternatively activated monocytes/macrophages (Mantovani et al., 2002, Gordon, 2003). The M1/M2 classification system in monocytes defines the distinct functional subsets of monocytes and parallels the Th1/Th2 paradigm described for T cells. M1 monocytes are characterized by the production of proinflammatory molecules like IL-12, IFNγ and TNFα in response to microbial agents and cytokines, where M2 monocytes exhibit anti-inflammatory properties and proangiogenic potential (Mantovani et al., 2002, Gordon, 2003). M2-polarized cells are not simply dampened M1 cells but they exhibit a distinct activation program when stimulated by variety of molecules including IL-4, IL-10, IL-13, glucocorticoid hormones and vitamin D3 to produce immunomodulatory cytokines like IL-10 (Mantovani et al., 2002, Gordon, 2003). Furthermore, alternatively activated M2 monocytes/macrophages are present in settings undergoing vascularization including tumors, endometriosis and wound healing (Kodelja et al., 1997, Mantovani et al., 2002, Sica et al., 2007, Bacci et al., 2009). The expression of CD163 and CD206 on Mf+ CD14+ IL-8+ and CD14+ IL-6+ cells suggests these cells exhibit characteristic features of alternative activation. On the other hand, the CD14+ monocytes producing IL-8 and IL-6 from Mf+ persons were also producing TNFα, a marker indicative of classical activation. Taken together, the presence of both markers for alternative and classical activation on the surface of these cells suggests the M1/M2 paradigm may be oversimplified.

Others have also found monocytes exhibiting characteristics of M1 and M2 cells simultaneously suggesting there are many factors contributing to the heterogeneity and activation state of monocytes and the subsets of monocytes/macrophages are still being continuously redefined (Gordon, 2003, Kreider et al., 2007, Umemura et al., 2008).

In rodent models, the presence and function of alternatively activated macrophages in filarial infection have been well-defined by their ability to suppress proliferation in many cell types and by their high level of expression of two macrophage genes, *Ym1 and Fizz1* also known as *Relmα* (Loke et al., 2000, 2002, Nair et al., 2003). Compared to rodent studies, human studies examining the role of alternatively activated macrophages in LF are limited due to the lack of well-characterized markers of alternative activation in humans and the lack of homologous molecules indicative of alternative activation between rodents and humans including an absence of human homologues for *Ym1* and *Fizz1* (Babu et al., 2009). In rodent studies of filariasis, alternatively activated macrophages produce elevated levels of IL-6 in response to worm infection (Loke et al., 2002). Babu et al. also demonstrated enhanced transcript levels of CD163 on cells from filarial-infected individuals (2009). Similarly, we have identified a cell type of the myeloid lineage in humans producing IL-6 in response to filarial ES products and also expressing M2 surface molecules, CD163 and CD206, indicative of alternative activation. Collectively, these data suggest the presence of a population of cells in humans with characteristics of pro-angiogenic alternatively activated M2 cells.

In conclusion, the lymphangiectasia seen in filarial-infected individuals may be due the sustained production of lymphangiogenic mediators by PBMCs in response to ES products during worm infection and monocytes, which are seen at higher frequencies in Mf+ persons, may be the cell type responsible for progression of subclinical lymphangiectasia toward lymphedema. With the completion of the *Brugia* genome and proteomic analyses of the *Brugia* secretome, we can begin to define the parasite products and molecular mechanisms leading the development of disease (Hewitson et al., 2008, Moreno and Geary, 2008, Bennuru et al., 2009). Understanding the mechanisms responsible for the early lymphatic dilation seen with filarial infection may help to provide a greater perspective on the molecules mediating lymphangiectasia and lymphangiogenesis in other conditions such as cancer and wound healing.

6.6 References

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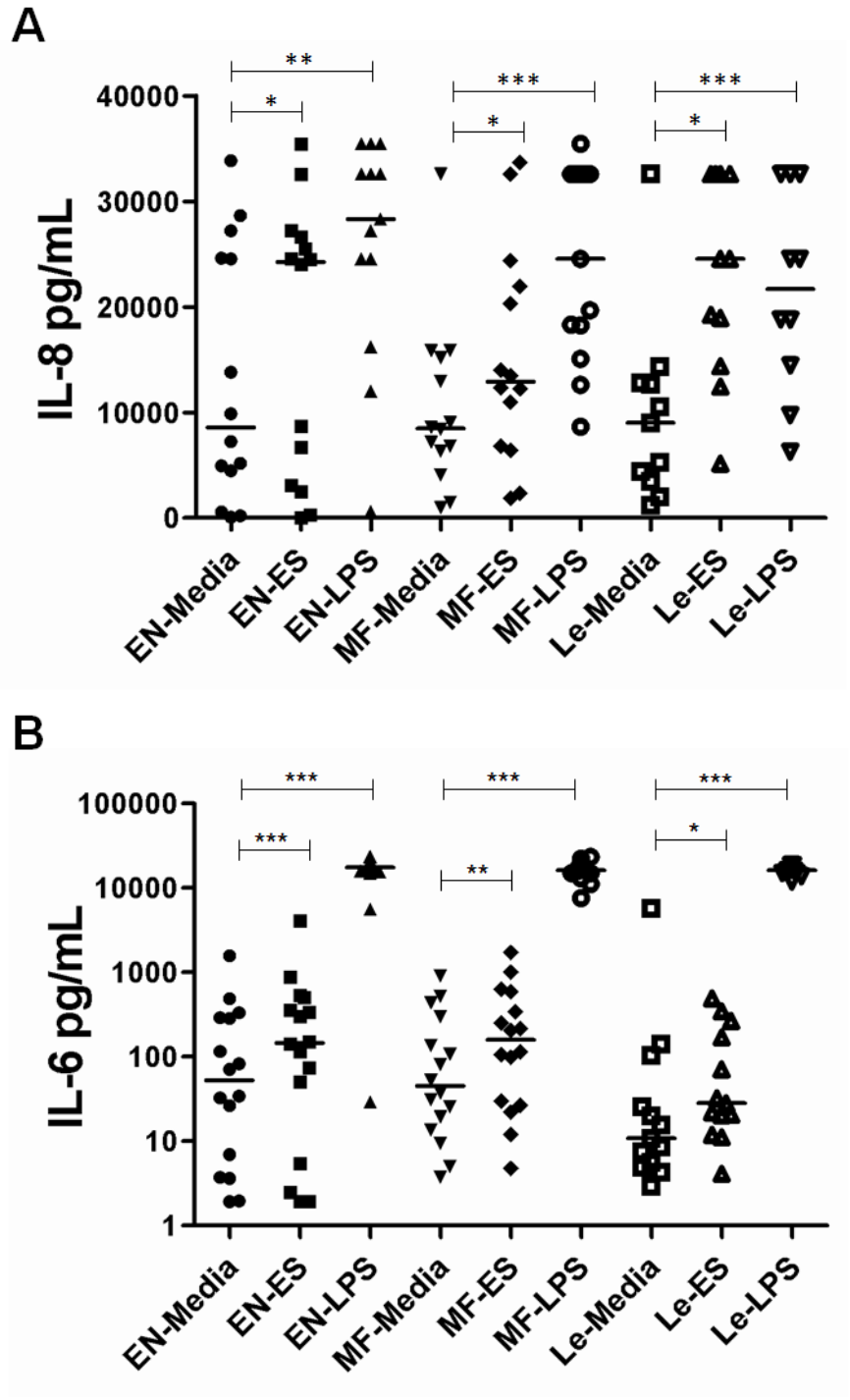
6.7 Figures and Tables

Figure 6.1. *Brugia* ES products induce the production of lymphangiogenic molecules by PBMCs from subjects in a filarial-endemic region. PBMCs were isolated from Mf+, Le and ENs from filarial-endemic regions of Haiti and $1x10^6$ cells were seeded and cultured with or without ES for 72 hrs. Cell supernatants were assessed for the presence of IL-8 (A), IL-6 (B), and VEGF-A (C) by luminex bead analysis. *Brugia* ES products induced the production of IL-8 (***$p<0.005$, **$p<0.007$, *$p<0.05$) and IL-6 (***$p<0.0005$, **$p<0.009$, *$p<0.05$) compared to cells in media alone as assessed by the Wilcoxon Signed Rank test. Mf+ persons produced more antigen-induced VEGF-A than ENs as compared by the Mann Whitney test (*$p<0.04$). Medians are presented as bars.

Figure 6.1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IL-8 pg/mL
40000
30000
20000
10000
0
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*
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*
EN-Media
EN-ES
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MF-Media
MF-ES
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Le-Media
Le-ES
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B
IL-6 pg/mL
100000
10000
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10
1
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EN-Media
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Le-ES
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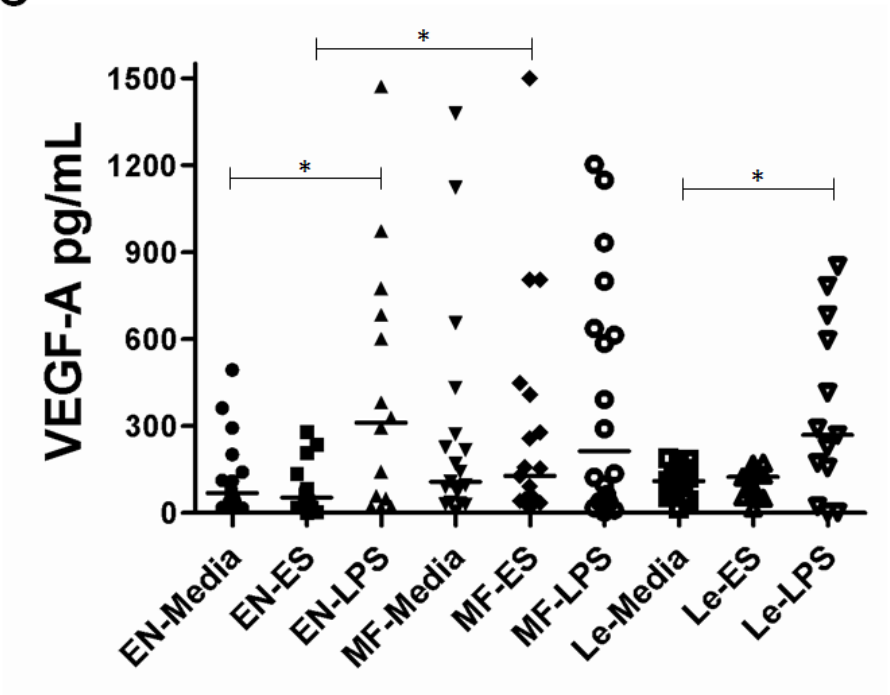
C


VEGF-A pg/mL
1500
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EN-Media
EN-ES
EN-LPS
MF-Media
MF-ES
MF-LPS
Le-Media
Le-ES
Le-LPS

Figure 6.2. Microfilaremic persons exhibit higher frequencies of monocytes producing lymphangiogenic mediators. PBMCs were isolated from Mf+ and ENs from filarial-endemic regions of Haiti and $5x10^5$ cells were seeded and cultured with or without ES. Cells were assessed for the presence of intracellular IL-8 (A) or IL-6 (B) by flow cytometric analysis. Monocytes were defined based on CD14 expression. (A) Representative flow cytometric data from one EN and one Mf+ individual with the percentage of IL-8+ monocytes in the upper right quadrant. (B) The percentage of IL-8+ monocytes across various individuals from both groups with each point representing one individual. The percentage of IL-8+ CD14+ monocytes in Mf+ were compared to EN persons by the Mann Whitney test ($p<0.04$). Medians are presented as bars. (C) Representative flow cytometric data from one EN and one Mf+ individual with the percentage of IL-6+ monocytes in the upper right quadrant. (D) The percentage of IL-6+ monocytes across various individuals from both patient groups with each point representing one individual. Medians are presented as bars.

Figure 6.2

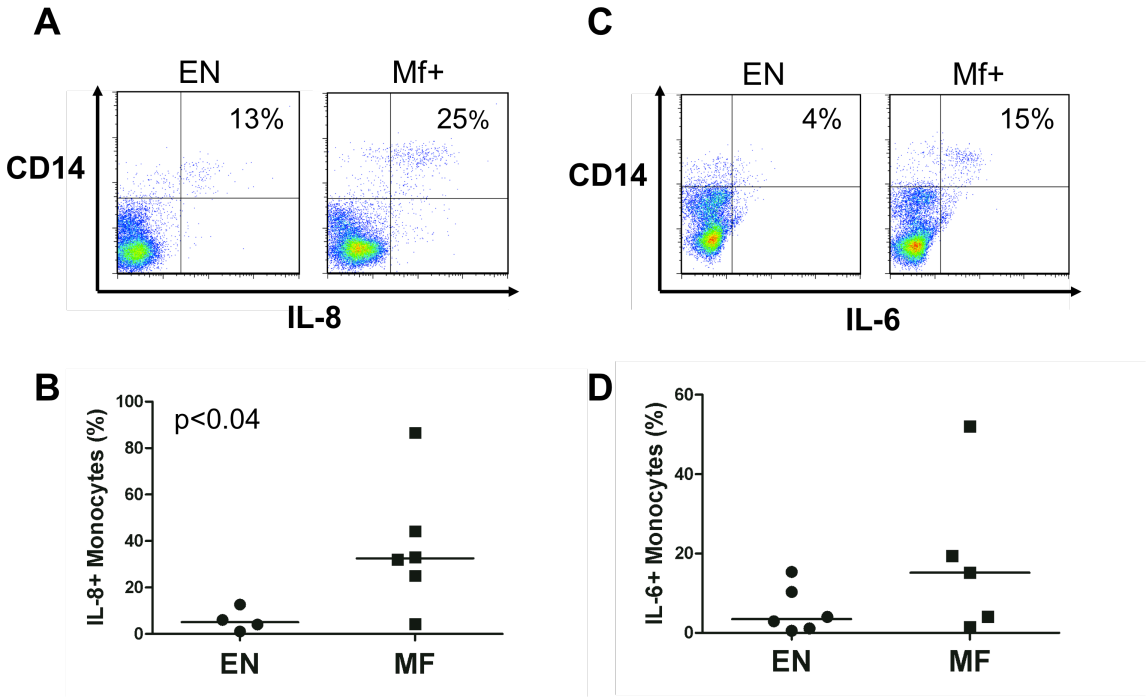
A
EN
Mf+
13%
25%
CD14
IL-8
C
EN
Mf+
4%
15%
CD14
IL-6
B
p<0.04
IL-8+ Monocytes (%)
100
80
60
40
20
0
EN
MF
D
IL-6+ Monocytes (%)
60
40
20
0
EN
MF

Figure 6.3. Phenotypic characterization of monocytes producing IL-8 and IL-6. PBMCs were isolated from Mf+ and ENs from filarial-endemic regions of Haiti and $5x10^5$ cells were seeded and cultured with or without ES. Cells producing IL-8 and IL-6 were assessed for the presence of CD68, CD163, CD206, HLA-DR and TNFα by flow cytometric analysis. (A and D) For our gating strategy, we first gated on all live cells, followed by the CD14+ IL-8+ (A) or the CD14+ IL-6+ (D) cells. Plots are not directly comparable to those in Fig. 6.2; a different antibody-fluorochrome conjugate was used for staining. (B and E) Histograms for expression of CD68, CD163, CD206, HLA-DR and TNFα on CD14+ IL-8+ (B) or CD14+ IL-6+ (E) cells. The solid black line in the histogram portrays one representative EN where the shaded grey area portrays one representative Mf+ person. The thin dotted black line shows control background staining. (C and F) Representative data showing the effects of ES-stimulation on CD14+ IL-8+ (C) or CD14+ IL-6 + (F) cells on CD68, CD163 and CD206 expression. The solid black line in the histogram portrays cells stimulated with ES where the dotted black line portrays cells from the same individual cultured in media alone.

Figure 6.3

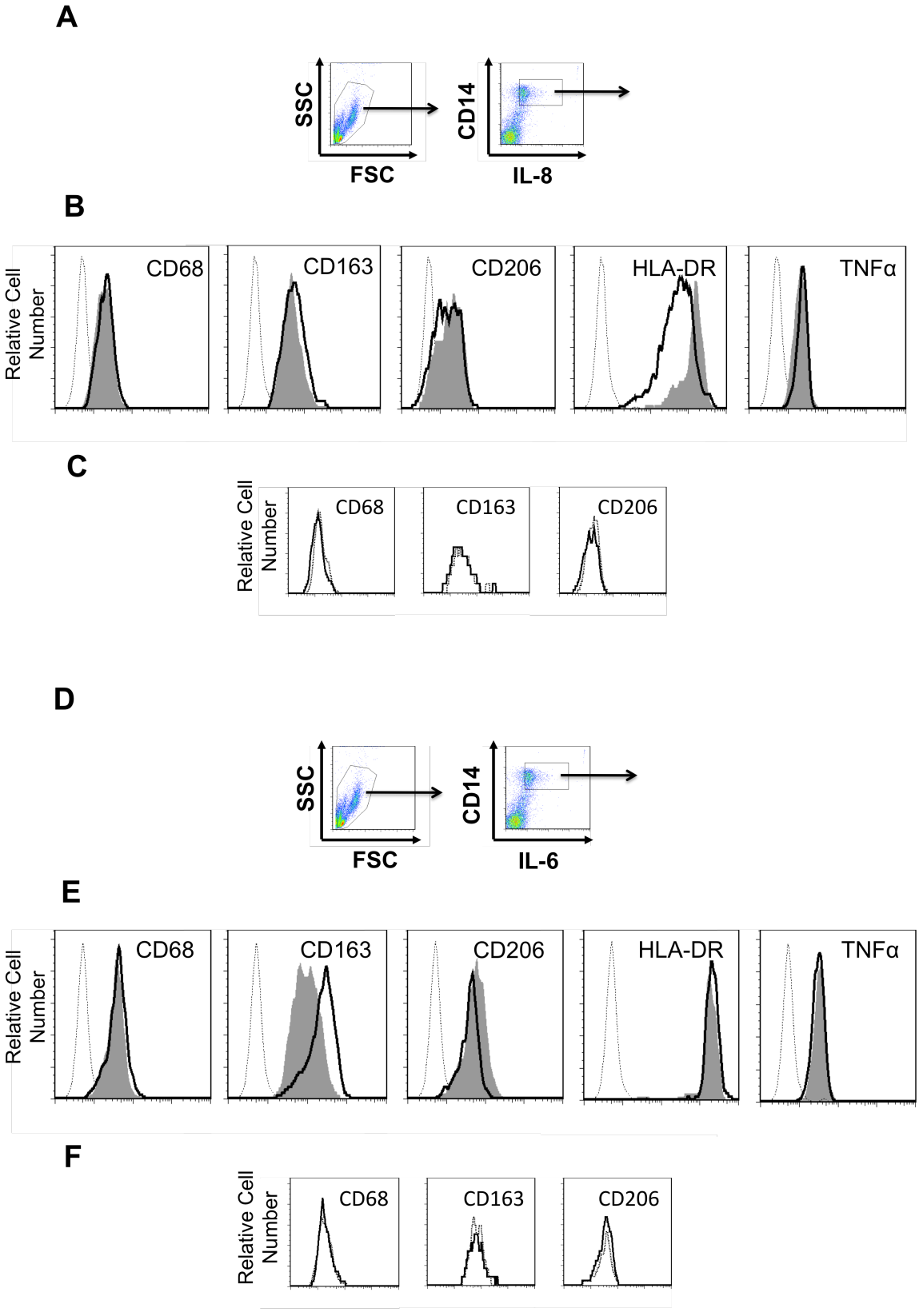
A
SSC
FSC
CD14
IL-8
B
Relative Cell Number
CD68
CD163
CD206
HLA-DR
TNFα
C
Relative Cell Number
CD68
CD163
CD206
D
SSC
FSC
CD14
IL-6
E
Relative Cell Number
CD68
CD163
CD206
HLA-DR
TNFα
F
Relative Cell Number
CD68
CD163
CD206

Table 6.1.
Phenotypic characterization of IL-8-producing monocytes from ENs and MF+[a]

	EN		MF+	
	Med[b]	ES[c]	Med[b]	ES[c]
CD68	16.2(12.0-20.8)	16.1 (12.9-17.8)	20.4 (11.9-38.4)	20.0 (9.8-36.1)
CD163	47.8 (30.2-77.1)	23 (17.9-26.8)	63.6 (16.4-158.0)	60.7 (17.1-133.0)
CD206	14.8 (9.5-18.4)	17.9 (15.2-19.9)	15.8 (11.5-20.6)	16.8 (12.2-22.9)
HLA-DR	934.8 (350.7-1835)		973.2 (240.8-1416)	
TNFα	20.4 (17.4-23.2)		20.6 (19.5-22.0)	

[a]$5x10^5$ PBMCs were stimulated with worm ES or cultured in media alone for 22 hours. Intracellular cytokines and cell surface molecules were analyzed by flow cytometry. Data are reported as the average median fluorescent intensities (MFI) for the group followed by the range of MFI across individuals.
[b]Cells from at least 6 different individuals per group (EN or MF) were cultured *ex vivo* and analyzed for the expression of CD68, CD163 and CD206. Cells from 4 different individuals per group were cultured and analyzed for HLA-DR and TNFα expression.
[c]Cells from 3 separate individuals per group were stimulated with ES, analyzed for the expression of CD68, CD163 and CD206 and compared to the MFI of cells cultured in media alone.

Table 6.2.
Phenotypic characterization of IL-6-producing monocytes from ENs and MF+[a]

	EN		MF+	
	Med[b]	ES[c]	Med[b]	ES[c]
CD68	30.4 (18.4-49.6)	18.6 (10.1-27.2)	28.5 (17.7-36.7)	25.3 (24.9-25.5)
CD163	144.2 (26.5-275.7)	42.0 (25.2-69.4)	97.0 (38.7-226.7)	71.5 (57.1-87.4)
CD206	50.8 (34.5-80.7)	87.5 (43.5-167.2)	44.1 (24.4-65.6)	41.6 (25.4-64.4)
HLA-DR	2093 (1891-2408)		1743 (1483-2089)	
TNFα	30.2 (28.2-32.4)		30.6 (27.0-33.1)	

[a]$5x10^5$ PBMCs were stimulated with worm ES or cultured in media alone for 22 hours. Intracellular cytokines and cell surface molecules were analyzed by flow cytometry. Data are reported as the average median fluorescent intensities (MFI) for the group followed by the range of MFI across individuals.
[b]Cells from at least 6 different individuals per group (EN or MF) were cultured *ex vivo* and analyzed for the expression of CD68, CD163 and CD206. Cells from 4 different individuals per group were cultured and analyzed for HLA-DR and TNFα expression.
[c]Cells from 3 separate individuals per group were stimulated with ES, analyzed for the expression of CD68, CD163 and CD206 and compared to the MFI of cells cultured in media alone.

CHAPTER 7

CONCLUSIONS: UNDERSTANDING THE MOLECULAR MECHANISMS OF LYMPHANGIECTASIA IN LYMPHATIC FILARIASIS[1]

[1]**Weinkopff, T., and P. Lammie. 2010. To be submitted to *Molecular and Biochemical Parasitology.***

Lymphatic filariasis (LF) is characterized by a wide spectrum of infection and disease. Individuals infected with the adult worms appear asymptomatic, but they do exhibit subclinical manifestations such as lymphangiectasia or lymphatic dilation (Amaral et al., 1994, Freedman et al., 1994, 1995, Noroes et al., 1996a, 1996b, Dreyer et al., 2000, 2002, Figueredo-Silva et al., 2002, Shenoy et al., 2008, 2009). At the other end of the spectrum of LF, individuals exhibiting clinical disease such as lymphedema are typically antigen-negative and devoid of adult worms (Figueredo-Silva et al., 2002). However, the natural history of lymphedema and its relationship to previous filarial infection remain uncertain. Not all filarial-infected individuals go on to develop lymphedema and we do not have a clear understanding of why only a subset of individuals living in an endemic area advance to clinical disease. It is important to understand the pathogenesis of disease since patients with lymphedema are typically not actively infected; consequently, treatment of infection does not reverse the pathology. Given the lymphatic dilation seen in infected vessels, we propose the dilation of these infected vessels may serve as an important risk factor for development of clinical lymphedema.

Several factors including both parasite and host factors may contribute to the development of disease. It has been proposed that parasite factors are responsible for the dilation of infected lymphatic vessels since removal of the worms with drug treatment reverses the subclinical pathology in both humans and infected animals; dilation is also seen in the SCID mouse model arguing that dilation is a result of the presence of the parasite and not the adaptive immune response (Vincent et al., 1984, Vickery et al., 1983, 1991, Nelson et al., 1991, Shenoy et al., 2008, 2009). The location of the worm may also

contribute to disease development since lymphedema occurs in many of the same anatomical locations associated with worm infection. In addition, host immunological factors may also contribute to the pathogenesis of disease. Patients with lymphedema exhibit heightened antifilarial immune reactivity with a robust Th1 profile including strong IFNγ and TNFα responses and elevated IgG1, IgG2 and IgG3 antifilarial antibodies compared to microfilaremic persons (Babu et al., 2009, Baird et al., 2002). On the other hand, microfilaremic individuals exhibit a pronounced immunomodulatory profile with impaired antigen-specific T- and B-cell responses, elevated IL-10 and IgG4 antifilarial antibodies (Ottesen et al., 1977, Piessens et al., 1980, Lammie and Katz, 1983, Nutman et al., 1987, Kurniawan et al., 1993, Mahanty et al., 1995, 1996, Yazdanbakhsh et al., 1995, Zhang et al., 1999, Baird et al., 2002, Nielsen et al., 2002a, 2002b). However, surprisingly both patients with microfilaremia and those with lymphedema possess similar antigen recognition profiles as defined by 2D immunoblots, suggesting specific antigens may not be associated with the development of disease (Weinkopff et al., 2009).

Host genetics may play a role in disease development since lymphedema clusters in high risk families and hereditary lymphedema has been associated with mutations in the *VEGFR-3* and *FOXC2* genes (Fang et al., 2000, Karkkainen et al., 2000, Cuenco et al., 2004). These data suggest that a subset of individuals may be predisposed to develop disease prior to worm infection. Secondary bacterial infections also play a role in progression of lymphedema to elephantiasis: 1) entry points for bacterial invasion are created by skin folds and lesions, 2) acute attacks related to bacterial infections exacerbate lymphedema, 3) the number of acute attacks correlates with disease severity,

and 4) patients with lymphedema also show heightened reactivity to bacterial superantigens and partial reversal of elephantiasis requires appropriate management of secondary bacterial infections (Dreyer et al., 1999, 2006, Gasarasi et al., 2000, Baird et al., 2002, McPherson et al., 2006). Taken together, many factors have been demonstrated to play a role in the development of lymphedema but we still have not defined the exact mechanisms responsible for the early lymphatic dilation seen in filarial-infected individuals and examined if this early dilation contributes to the development of lymphedema.

Dilation is detected along the length of the vessel and is not just restricted to the site of the worm nest arguing a soluble factor released by the worm that can travel the length of the vessel may be contributing the lymphatic pathology (Amaral et al., 1994). Given the close relationship between the parasite and the lymphatic endothelium and the dilation of infected lymphatic vessels (LV), we hypothesized that filarial ES products were responsible for the lymphangiectasia seen in infected vessels. Our working model can be seen in Figure 7.1. We hypothesized that the parasite alters the lymphatic endothelial cells (LEC) which initiates the development of disease. However, worm excretory-secretory (ES) products do not appear to directly activate LECs based on the lack of LEC proliferation, growth factor and cytokine production as well as the lack of changes seen in cell surface molecule expression by LECs in response to filarial ES products (Chapter 4). Therefore, we examined the potential of an indirect activation of LECs by an accessory cell type and found that peripheral blood mononuclear cells (PBMC) secrete cytokines and members of the vascular endothelial growth factor (VEGF) family in response to ES products. Many of the molecules we detected in

response to filarial ES products such as IL-8, IL-6 and VEGF-A have been shown to induce endothelial cell proliferation, migration and differentiation. Furthermore, fractionation experiments showed that CD14+ monocytes are the primary producers of IL-8 and VEGF-A in response to worm ES products, but CD14-depleted populations produce measurable quantities of IL-6. We next tested the ability of these molecules to induce the differentiation of the LECs as measured by tubule formation. Concentrations of IL-8, IL-6 and VEGF-A secreted by PBMCs in response to *Brugia* ES can induce tubule formation by LECs *in vitro*. Parallel studies using Matrigel plugs in an *in vivo* rat model revealed the formation of blood and lymphatic vessels in response to IL-8, IL-6 and VEGF-A. Supernatants from human PBMCs stimulated with ES products which include measurable concentrations of IL-8, IL-6 and VEGF-A also stimulated blood and lymphatic vessel formation in the rat *in vivo*. Taken together, these findings establish the ability of the lymphangiogenic molecules IL-8, IL-6 and VEGF-A which are released in response to worm ES products to alter LEC phenotype.

Once we characterized the responses of filarial-naïve individuals in response to filarial ES products, we wanted to measure production of these lymphangiogenic molecules by cells from individuals living in a filarial-endemic region. Haitian PBMCs stimulated with worm ES products produced increased levels of IL-8 and IL-6. Following the identification of monocytes as the cellular source for these molecules in naïve uninfected individuals, we examined the CD14+ monocytes in Haitians living in LF-endemic regions. Microfilaremic individuals exhibit higher frequencies of CD14+ IL-8+ cells compared to endemic normal individuals living in the same endemic area. These IL-8- and IL-6-producing monocytes also expressed CD68, CD163 and CD206.

Others have also reported elevated transcript levels of CD163 on cells from filarial-infected individuals (Babu et al., 2009). CD68 is macrophage differentiation marker confirming the monocyte/macrophage lineage of the CD14+ monocytes. CD163, a cysteine-rich group B scavenger receptor, and CD206, a mannose receptor, have been shown to be markers of M2-polarized monocytes/macrophages, also termed alternatively activated monocytes/macrophages (Mantovani et al., 2002, Gordon, 2003).

The expression of CD163 and CD206 by the IL-8- and IL-6-producing monocytes from filarial-infected individuals suggests that these cells show characteristics of M2 or alternatively activated monocytes/macrophages. Compared to the proinflammatory M1 monocytes, which are characterized by the production of proinflammatory molecules like IL-12, IFNγ and TNFα in response to microbial stimuli and cytokines, the M2 monocytes/macrophages exhibit a distinct activation program when stimulated by IL-4, IL-10, IL-13, glucocorticoid hormones or vitamin D3 to produce immunomodulatory cytokines such as IL-10 (Mantovani et al., 2002, Gordon, 2003). M2, or alternatively activated, monocytes/macrophages exhibit anti-inflammatory properties and pro-angiogenic potential and have been shown to play a role settings undergoing vascularization such as tumorigenesis, wound healing, endometriosis and chronic inflammation (Kodelja et al., 1997, Mantovani et al., 2002, Sica et al., 2007, Bacci et al., 2009).

Alternatively activated macrophages have been described in filarial infections of humans and animals (Loke et al., 2000, 2002, Nair et al., 2003, Babu et al., 2009). The alternatively activated macrophages in rodent filarial models are also producing elevated levels of IL-6, similar to the monocytes we have characterized in human filarial

infections, but the lack of homologous molecules indicative of alternative activation between humans and rodents makes it difficult to draw exact parallels between the two cell populations (Loke et al., 2002). Furthermore, alternatively activated macrophages are thought to contribute to the overall immunosuppression associated with filarial infection; however, it is interesting that this is the same cytokine milieu that provides an environment favoring the development of lymphangiectasia, suggesting that lymphangiectasia occurs in a setting where the parasite is actively modulating the immunologic environment towards a Th2 setting. The presence in filarial-infected individuals of increased frequencies of monocytes producing molecules with lymphangiogenic potential suggests the parasite is responsible for inducing this host response. The parasites' ability to modulate the host response has been widely demonstrated; it has been hypothesized that the parasite orchestrates an anti-inflammatory response to evade the immune system and encourage parasite persistence and thus the transmission of the organism. The M2-biased cell populations described here, and the well-characterized Th2-biased cytokine environment, are both induced by the infection and may work in parallel to intensify immune evasion mechanisms and create a niche for the worm in the dilated LVs that is rich in nutrients and resources due to the decreased lymphatic flow associated with dilation.

In conclusion, these data support the hypothesis that *Brugia* ES products activate monocytes, which could modulate lymphatic function and contribute to lymphangiectasia and thus the development of disease. The production of lymphangiogenic molecules including IL-8, IL-6 and VEGF-A is elevated in response to filarial ES products and these molecules can induce tubule formation *in vitro* and *in vivo*. The presence of these

molecules and monocytes producing these factors are also seen in filarial-infected individuals. Typically, lymphangiogenic molecules are thought to be beneficial in wound healing and lymphedema models due to their support of the lymphatic architecture (Karkkainen et al., 2001, Szuba et al., 2002, Yoon et al., 2003, Galiano et al., 2004), but the sustained production of these molecules over the course of a filarial infection maybe deleterious for the lymphatic endothelium. The cumulative effects of these lymphangiogenic factors over time may lead to defective lymphatic dysfunction and contribute to the development of lymphedema (Jeltsch et al., 1997, Nagy et al., 2002, Angeli and Randolph, 2006). Defining the role of monocytes and the lymphangiogenic factors in the early lymphatic dilation seen in filarial-infected individuals may provide a better understanding in the factors contributing to the pathogenesis of disease.

The working model seen in Fig. 7.1 demonstrates a complex relationship between filarial ES products, CD14+ monocytes and lymphangiogenic mediators. The model may help to explain the molecular mechanisms involved in the lymphangiectasia seen in infected individuals but the relationship between lymphangiectasia and lymphedema still remains unclear. Therefore, the question of why only a small proportion of individuals go on to develop disease still needs to be addressed. The body of work described here attempts to define the molecular mechanisms contributing to lymphangiectasia but future work must determine if varying degrees of lymphangiectasia as assessed by imaging technologies correlate with increased production of lymphangiogenic mediators. These experiments may provide the link between lymphangiectasia and the development of lymphedema.

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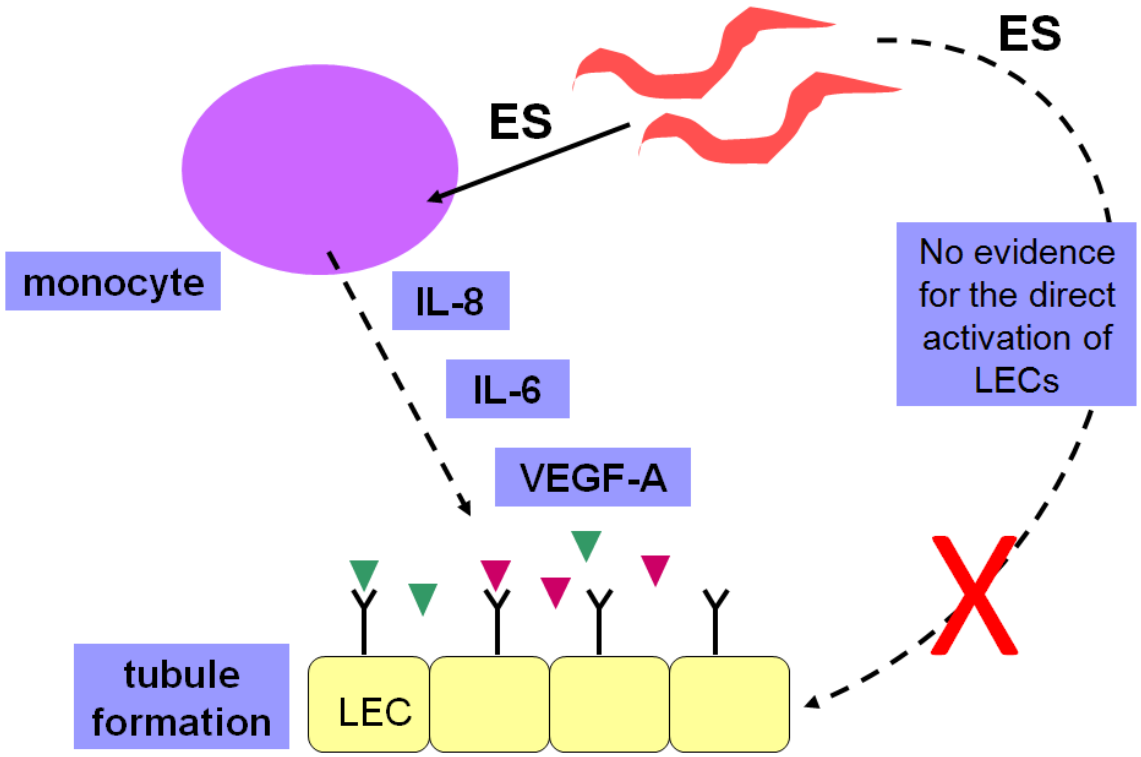
7.3 Figure

Figure 7.1. *Brugia* ES products activate monocytes which may modulate LEC function. Filarial worms secrete ES products which do not appear to directly activate LECs; however, *Brugia* ES products activate monocytes by inducing the production of IL-8 and IL-6 and VEGF-A (our data depicted in blue boxes) and that monocytes are the primary producers of IL-8 and VEGF-A. These lymphangiogenic molecules stimulate LEC differentiation by inducing tubule formation *in vitro* and *in vivo*. The action of these factors on LECs may contribute to lymphangiectasia and thus the pathogenesis of filarial lymphedema.

Figure 7.1


ES
ES
monocyte
IL-8
IL-6
VEGF-A
No evidence for the direct activation of LECs
tubule formation
LEC