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MONOCYTES AND MACROPHAGES AS DETERMINANTS OF SUSCEPTIBILITY TO INFECTION IN LYMPHATIC FILARIASIS

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ABSTRACT

TITLE: Monocytes and macrophages as determinants of susceptibility to infection in lymphatic filariasis

BACKGROUND & AIMS: Helminths induce strong regulatory and T helper 2-type responses by targeting host cells. In lymphatic filariasis, the host response to this, together with a multitude of other factors, determines whether a person remains infection and disease free or develops a successful infection. Infection results in either asymptomatic infection, which benefits transmission of the parasite, or chronic pathology, which is responsible of the high levels of morbidity seen in patients. Monocytes and macrophages contribute to helminth-induced dysfunction of the immune response through modulation by microfilariae in the blood and tissues. During patent infection monocytes encounter microfilariae in the blood, an event that occurs in asymptomatically infected patients who are immunologically hyporeactive. Furthermore helminths induce regulatory antibody responses that may impact on disease outcome. Other disease models have shown that altered glycosylation of the IgG Fc region correlates with pathology, whereby decreased galactosylation is associated with inflammation and increased sialylation is associated with anti-inflammatory responses. The aim of this project was to determine whether microfilariae act on blood monocytes and macrophages to induce a regulatory phenotype that interferes with innate and adaptive responses. Furthermore the IgG glycosylation profile of the different disease outcomes was compared with determine a role for glycosylation in lymphatic filariasis.

PRINCIPAL FINDINGS: Monocytes and *in vitro* generated macrophages from filaria non-endemic normal donors stimulated with *Brugia malayi* microfilarial (Mf) lysate but not adult female lysate show a drastically altered phenotype. Monocytes stimulated with Mf lysate develop a defined regulatory phenotype, characterised by expression of IL-10 and PD-L1. Importantly, this regulatory phenotype was recapitulated in monocytes from *Wuchereria bancrofti* asymptomatically infected individuals but not patients with pathology or endemic normals. Monocytes from non-endemic donors stimulated with Mf lysate directly inhibited $CD4^+$ T cell proliferation and cytokine production. $CD4^+$ T cell IFN-γ responses were restored by neutralising IL-10 or PD-1. Furthermore, macrophages stimulated with Mf lysate expressed high levels of IL-10 and had suppressed phagocytic abilities. Finally Mf lysate applied during macrophage differentiation *in vitro* selectively interfered with macrophage abilities to respond to LPS stimulation. Additionally, Fc region *N*-linked glycans of total IgG from *W. bancrofti*-exposed donors were analysed. Using capillary electrophoresis it was found that there was no difference in galactosylation of total IgG between the different disease outcomes, however, asymptomatically infected patients had significantly lower levels of disialylated IgG compared with endemic normals and patients with pathology.

CONCLUSIONS & SIGNIFICANCE: Conclusively, this study demonstrates that Mf lysate stimulation of monocytes from healthy donors *in vitro* induces a regulatory phenotype, able to interfere with $CD4^+$ T cell responses. This phenotype is directly reflected in monocytes from filarial patients with asymptomatic infection but not patients with pathology or endemic normals. The results suggest that suppression of T cell functions typically seen in lymphatic filariasis is caused by microfilaria-modulated monocytes in an IL-10- or PD-1-dependent manner. Together with suppression of macrophage innate responses, this may contribute to the overall down-regulation of immune responses observed in asymptomatically infected patients.

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LIST OF ABBREVIATIONS

AAM	alternatively activated macrophage
AMCase	acidic mammalian chitinase
ACN	acetonitrile
APC	antigen-presenting cell
APTS	9-aminopyrene-1,3,6-trisulfonic acid
Arg	arginase
AS	asymptomatically infected
AvCystatin	cystatin from *Acanthocheilonema viteae* (= Av17)
Bm-CPI-2	cystatin from *Brugia malayi*
BSA	bovine serum albumin
CCL	chemokine ligand
CCR	chemokine receptor
CE-LIF	capillary electrophoresis laser-induced fluorescence
CFA	circulating filarial antigen
CFSE	carboxyfluorescein succinimidyl ester
CP	chronic pathology
CTLA	cytotoxic T-lymphocyte antigen
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DEC	diethylcarbamazine
ELISA	enzyme-linked immunosorbent assay
EN	endemic normal
EU	endotoxin units
F	female
GITR	glucocorticoid-induced TNFR-related protein
GM-CSF	granulocyte macrophage colony-stimulating factor
hs	hours
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
LPS	lipopolysaccharide
LSM	lymphocyte separation medium
M	male
MAP	mitogen-activated protein
M-CSF	macrophage colony-stimulating factor
Mf	microfilariae
MHC	major histocompatibility complex
min	minutes
MIF	macrophage migration inhibitory factor
MRC	mannose receptor C type
ND	not detected
NO	nitric oxide
ns	not significant
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PD-L	programmed death-ligand
R	receptor
r	recombinant
RELM	resistin-like molecule

RT-PCR	realtime polymerase chain reaction
SEA	*Schistosoma mansoni* soluble egg antigen
sec	seconds
SEM	standard error of the mean
TFA	trifluoroacetic acid
TGF	transforming growth factor
THF	tetrahydrofuran
TNF	tumour necrosis factor
Th	T helper
TLR	Toll-like receptor
Treg	T regulatory

1. INTRODUCTION

1.1. Lymphatic filariasis

Filarial nematodes are helminths that produce chronic infection in humans and cause suffering and debilitating disease. In lymphatic filariasis, the filariae reside in the lymphatics to cause chronic infection. An estimated 120 million people worldwide are infected; 90% of these infections are caused by the species *Wuchereria bancrofti*, while the remainder are caused by *Brugia malayi*, and to a lesser extent, *B. timori*[1]. While mortality is rare, morbidity is extremely high and results in permanent and long-term damage, characterised by destruction of the lymphatic vessels[2]. Lymphatic filariasis is found in 83 countries worldwide, predominantly in Africa and Asia. A mosquito vector transmits the disease, and the vector species varies depending on the geographical location[3]. *W. bancrofti* is carried by vectors of the species *Culex*, *Anopheles* or *Aedes*, which are most common in the Americas, Africa and Asia, respectively. In contrast *Mansonia* is the main vector for Brugian parasites.
The lifecycle for Brugian and Bancroftian parasites is very similar. Only Brugian species have known animal (feline and primate) reservoirs. An example of the lifecycle for lymphatic filaria is shown in **Figure 1-1**. The microfilarial lifecycle stage displays periodicity that is dependent on the blood feeding patterns of the vector species present in the relevant geographical location. Thus *W. bancrofti* microfilariae, for example, display predominantly nocturnal periodicity, such that they are detected in the bloodstream only during the approximate hours of 21:00 to 04:00. An exception is the Pacific Islands where microfilariae are found in the bloodstream continuously but in varying density depending on the time of day[4,5]. When microfilariae are not in the blood, they sequester deep in the tissues, particularly the lungs[6-9].

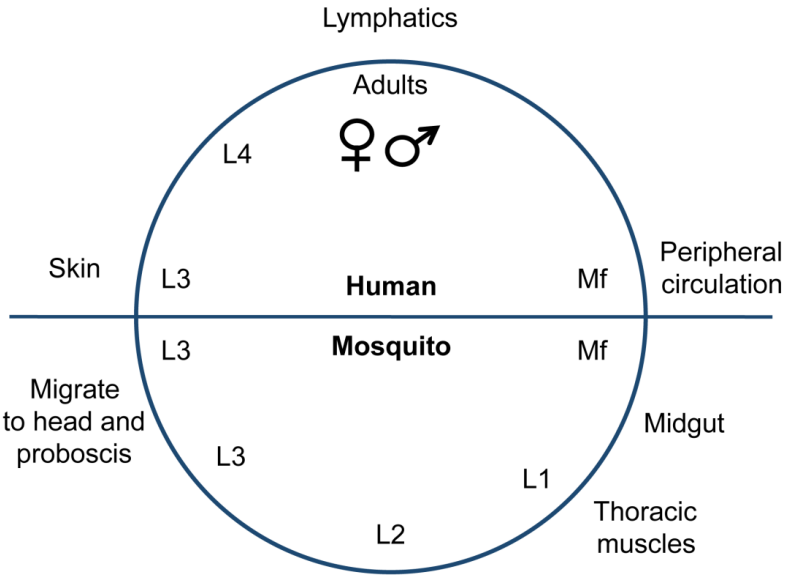


Figure 1-1. The lifecycle of lymphatic filarial worms.
Human infection occurs when a mosquito carrying the L3 infective larvae takes a blood meal. L3 larvae enter the blood and travel from the skin to the lymphatics to develop into adults that produce microfilariae (Mf) over a period of 6-12 months. Microfilariae circulate between the blood and the lymphatic system. While in the blood, microfilariae are picked up by a mosquito taking a blood meal, and thus are transmitted to the next individual. In the mosquito, microfilariae undergo further development over 10-14 days, from the L1 to the L3 stage, thereby maintaining the lifecycle (adapted from elsewhere[10,11]).

No vaccine currently exists on the market for the treatment or prevention of filarial infection. The main method of control used is to target disease transmission between host and vector. This is achieved by annual or biannual mass drug administration of chemotherapeutic drugs, and was initiated with the introduction of diethylcarbamazine (DEC) rapidly after the drug's anti-filarial effects were discovered in 1947[12,13]. The current protocol is to provide DEC or ivermectin combined with albendazole to reduce the levels of microfilariae in the blood for a period of six months or more. This aims to decrease the number of microfilaria-positive cases to such an extent that transmission is prevented[14]. These drugs are predominantly microfilaricidal with only moderate macrofilaricidal effects and therefore must be administered for a number of years until the adult worms die naturally[14]. New drugs such as the tetracycline antibiotic doxycycline are being developed to tackle this issue. Doxycycline depletes the obligate mutualistic endosymbiont *Wolbachia* that is carried by Bancroftian and Brugian parasites and leads to long-term worm sterility and death[15]. Furthermore, treatment partially reverses pathology in patients who already have established lymphatic disease[16]. Thus *Wolbachia* depletion appears a promising candidate to treat infection and to reverse established pathology.

1.2. The immune response in filarial infection

Parasitic filariae are multicellular organisms that display longevity in the host and have complex lifecycles. Thus a unique evolutionary relationship has developed between these parasites and the immune system of the host. The next section will examine the diverse immune responses that develop in filarial infection. An overview is provided of the innate and adaptive immune responses induced by the host to resist infection, and the parasite-induced mechanisms that have evolved to modulate and circumvent these responses, ultimately leading to persistence of the parasites within the host.

1.2.1. The spectrum of clinical outcomes in filarial infection

In areas endemic for lymphatic filariasis it has been established that not all infected individuals develop pathology. It has long been recognised that helminths induce a hyporesponsive immune phenotype in the majority of persons that allows the establishment of infection while simultaneously preventing or reducing signs of disease in the host[17]. In fact, antigen-specific cellular hyporesponsiveness was already described for filarial infections more than 30 years ago[18]. Lymphocyte proliferation levels in adults infected with *W. bancrofti* were compared with endemic normal controls who were negative for all signs of infection or disease. Infected individuals had significantly lower levels of proliferation in response to filarial antigen compared with endemic normal controls[18]. Subsequently the distinction was made between microfilaria-positive asymptomatically infected persons, microfilaria-negative patients showing clinical symptoms of lymphatic filariasis and endemic normal controls who carried no signs of infection or disease[19]. It was suggested that the outcome of disease depends on a multitude of factors, including parasite-induced immunoregulation and host genetic background (reviewed elsewhere[20]). Epidemiological studies of helminth infections thus recognise two main, distinct clinical outcomes that depend on immune regulation induced by the parasite together with the genetic background of the host. These two outcomes include chronic pathology and asymptomatic infection, and are found alongside infection-free, endemic normal individuals (**Figure 1-2**, reviewed elsewhere[21]). A third but rare manifestation of the disease exists in individuals infected with *W. bancrofti* and *B. malayi*, and is known as tropical pulmonary eosinophilia, characterised by coughing and wheezing as well as high levels of blood eosinophilia and serum immunoglobulin (Ig) E[22].

The overt manifestation of chronic lymphatic pathology (abbreviated as CP) is caused by a hyperresponsive phenotype that develops in patients. These individuals have increased antigen-specific IgE and low IgG4[23,24], they develop strong T helper (Th)1 and Th17 pro-inflammatory responses and a greatly diminished T regulatory (Treg) compartment[23], resulting in immunopathological changes in the host. CP patients carry adult worms in the lymphatics and are generally amicrofilaremic. Parasite death leads to the release of antigenic material that triggers inflammation and causes destruction of lymphatic vessels[22]. In *W. bancrofti, B. malayi* and *B. timori* infections, this can result in the development of lymphedemas and ultimately elephantiasis or hydrocoele, whereby the lymphatic tissue becomes dilated and hypertrophic.

The second manifestation is a hyporesponsive phenotype characterised by subclinical or asymptomatic infection (abbreviated as AS), which tolerates the presence of fecund adult worms as a result of strong parasite-induced immunosuppression[25]. Importantly, adult worms are tolerated and circulating blood microfilariae are carried by these patients, ensuring transmission. This group has increased numbers of regulatory cells, high interleukin (IL)-10 and elevated levels of antigen-specific IgG4 leading to a modified Th2 response that protects the host from immunopathology and permits parasite survival[25] (see below for a more detailed description of the modified Th2 response). Thus, parasite-induced immunomodulation allows persistent infection and continuous transmission while simultaneously enabling the host to tolerate infection by diminishing clinical symptoms.

The proportion of individuals in a filaria-endemic area who do not develop one of these two clinical manifestations remain infection- and disease-free and are putatively immune; these

individuals are known as endemic normals (abbreviated as EN)[21]. Thus a fine balance of different aspects of immunity is required to develop a response beneficial to both the host and the parasite.

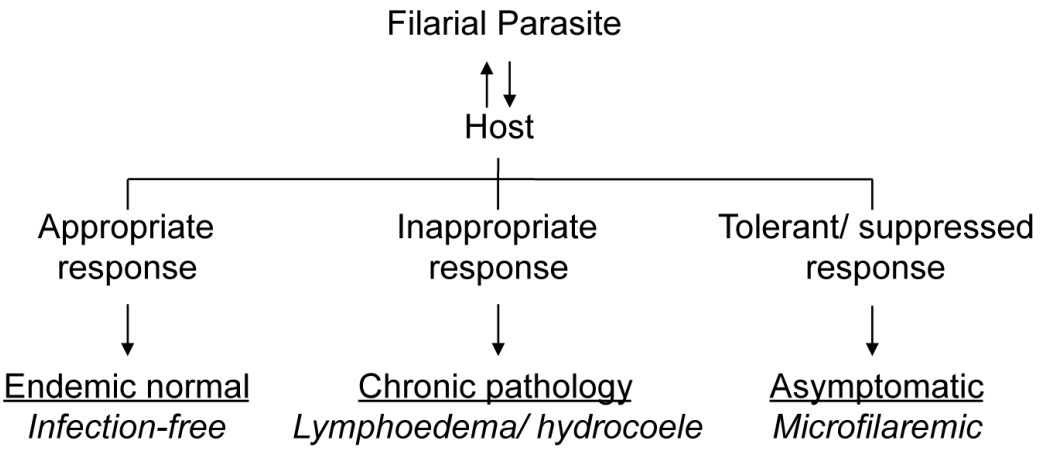


Figure 1-2. The spectrum of clinical outcomes observed in filarial infection.
Adapted from elsewhere[17].

There is currently no murine model of lymphatic filariasis that directly reflects this spectrum of disease. The lifecycles of *B. malayi*, *B. pahangi* and *Acanthocheilonema viteae* can be maintained in Mongolian jirds (*Meriones unguiculatus*); similarly *B. pahangi* can be maintained in rats and even results in lymphatic pathology[26]. However, currently the choice of immunological tools for these models is very limited. Different stages of the *B. malayi* lifecycle can be implanted into mice to investigate the immune response to these specific stages; however, patent infection does not develop[27]. *Litomosoides sigmodontis* productively infects mice, but does not cause lymphatic pathology. Finally there are strains of mice that are submissive to full patent infection and develop lymphatic pathology, for example severe combined immunodeficiency (*scid*/*scid*) mice, which have no T or B cells[28]. However, these strains may not fully represent real infection or immune responses in humans. This makes comparisons between mice and humans particularly difficult. Therefore current filarial research has two foci: (i) human blood-derived cellular or humoral research that provides an indication of processes occurring deep in the tissues and lymphatics, and (ii) rodent-based research that attempts to extract and translate evidence to the human system, bearing in mind the caveats mentioned in using these models. Nevertheless murine research provides a good basis for the understanding of individual immune mechanisms involved in filarial infection, which together with human blood-derived research presents a concise picture of the immune response to filariasis in humans[27].

1.2.2. Host protection in helminth infection

Host protection against invading helminths is defined by a strong Th2-type immune response that destroys and/or expels the parasite. Cells of the innate and adaptive immune system are important for initiation of Th2-type immunity. Th2-type immunity involves $CD4^+$ Th2 cells and the cytokines IL-4, IL-5, IL-10, and IL-13, the antibody isotype IgE as well as the chemokine ligand CCL11 and its receptor CCR3[21,29,30]. The Th2 response is in clear contrast to the Th1 response that typically develops against bacterial or viral challenge, and these two responses that lie on opposite ends of a spectrum are usually mutually exclusive. The initiation of a Th2 response leads to recruitment and infiltration of eosinophils, basophils and mast cells, and differentiation of alternatively activated macrophages[31]. Notably, Th2-type immune responses highlight three major features in helminth infection: inflammation, wound repair and most importantly, parasite resistance.

1.2.2.1. Evidence of immunity in humans

Immunity to parasitic filariasis in humans has been described in the form of endemic normals who live in a filaria-endemic region and have the same level of exposure to the parasite as patients with pathology or asymptomatic infection[32]. Reports illustrate that endemic normals typically have a mixed Th1/Th2 response, strong $CD4^+$ T cell proliferative responses, and a low ratio of antigen-specific IgG4 to IgE. Turaga *et al.* demonstrated that putatively immune endemic normals have high levels of the Th2 cytokine IL-5 and the Th1 cytokine interferon (IFN)-γ in response to peripheral blood mononuclear cell (PBMC) stimulation with L3 larval and adult male antigens from *Onchocerca volvulus*, a tissue-dwelling filarial species[33]. Another study reported that stimulation of PBMCs from endemic normals with an *O. volvulus* L3-derived protein induced T cell proliferation and significant levels of IL-5 and IFN-γ, but little induction of IL-4[34]. Interestingly, these individuals did not have antibodies specific to the L3 protein, suggesting that B cells play a minimal role in protection. High levels of IFN-γ and IL-5 have also been reported elsewhere after stimulation of PBMCs from endemic normals with *O. volvulus* adult worm antigen[35]. In *W. bancrofti* infection, PBMCs from endemic normals, in contrast to PBMCs from microfilaremic patients, proliferate strongly and produce high amounts of IFN-γ in response to stimulation with *B. malayi* adult antigen[36]. This was also demonstrated in an earlier study where PBMCs from *W. bancrofti*-exposed endemic normal donors proliferated strongly, and produced high levels of IFN-γ, IL-2, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF) in response to stimulation with *B. malayi* adult or micofilarial antigen[37]. Again, no IL-4 could be detected in this study[37].
In the last decade the coverage of mass drug administration in filaria-endemic regions, particularly in India, has worked extremely efficiently, resulting in very low levels of *W. bancrofti* and *B. malayi* in these areas[38,39]. Thus whether endemic normal individuals studied today truly display immunity and are able to eliminate L3 larvae upon initial infection, or whether this population has actually never been exposed is not clear.

1.2.2.2. Evidence of immunity from animal models

The need to understand immunity against helminths is driven by the hope of developing a vaccine against lymphatic filariasis. As yet, no vaccine exists for humans; however, animal studies using irradiated L3 larvae have demonstrated some level of protection. L3 larvae induce Th2 responses in murine models, characterised by production of IL-4 from $CD4^+$ T cells[40]. Vaccination with irradiated L3 larvae from *O. volvulus* has been shown to lead to partial protection in mice against challenge infection[41]. Furthermore it could be shown that this protection was entirely dependent on the presence of eosinophils and IgE[42]. A strong IgE response has also been reported in jirds infected with *A. viteae*, supporting the evidence for a role of Th2 responses in immunity[43].
The filarial species *B. malayi*, *W. bancrofti* and *O. volvulus* do not develop patent infection in mice; therefore studies have focused on murine models that reflect the human diseases. BALB/c mice are naturally susceptible while C57BL/6 mice are resistant to infection with the murine parasite *L. sigmodontis*[44]. In resistant strains, the role of IL-4 has been demonstrated as essential in providing protection, as removal of this cytokine leads to susceptibility to *L. sigmodontis* in C57BL/6 mice[45]. However, the same research group highlighted the fact that BALB/c mice, which display susceptibility to infection, also produce high levels of IL-4 together with a strong Th2 response[45]. Following from this, it could be shown that susceptible strains rapidly develop a population of Treg cells expressing Foxp3 upon infection that are essential to allow establishment of patent infection[46]. Ablation of this regulatory population did not affect larval numbers but significantly inhibited adult worm development, fecundity and blood microfilaria levels[46]. This Treg cell population expressed the IL-2 receptor CD25, and the regulatory markers cytotoxic T-lymphocyte antigen (CTLA)4 and glucocorticoid-induced TNFR-related protein (GITR) and was hyporesponsive in terms of proliferation and cytokine production[47]. Finally, neutralisation of CD25 and GITR converted the otherwise susceptible mouse strain into a resistant phenotype[47].

In summary, murine models have contributed a great deal to the understanding of protection and immunity in the host, and have allowed the dissection of specific cell populations and cytokine responses that are involved in these processes.

1.2.3. Helminth-derived immune regulation of the host response

Helminths have evolved various strategies to modulate host responses and ultimately suppress host-protective Th2-type immunity to enable their own survival by inducing innate and adaptive regulatory cells and specific inhibitory antibody isotypes (reviewed elsewhere[29]). This is thought to ensure long-term persistence of the parasite in the host. Immunomodulation occurs through production of specific parasite-derived products that target mammalian host immune cells and signalling pathways, inducing a highly directed host response known as a "modified Th2 response". This response is beneficial for the host as it limits Th2-associated inflammation[21]. This is strictly dependent on live parasites as shown by the recovery of cellular responsiveness in patients treated with microfilaricidal chemotherapy, specifically DEC[48]. While some adults are killed by DEC treatment, the main target is the microfilarial stage, suggesting a prominent role for microfilariae in modulating immune responses[2]. In immunological terms, the modified Th2 response is defined by the development of specific antibody isotypes, including induction of IgG4 (in humans) accompanied by a decrease in IgE, IL-4 and IL-5, while IL-10 levels from different regulatory cell sources increase[49]. These mechanisms attenuate pathology, induce tolerance, and ultimately allow persistence of the worm. This results in asymptomatic infection and sustains parasite feeding, successful reproduction and continuation of the parasite lifecycle[21,49]. **Figure 1-3** demonstrates the host-derived protective responses that are induced in helminth infection, and the cellular targets of helminth-induced immune modulation.

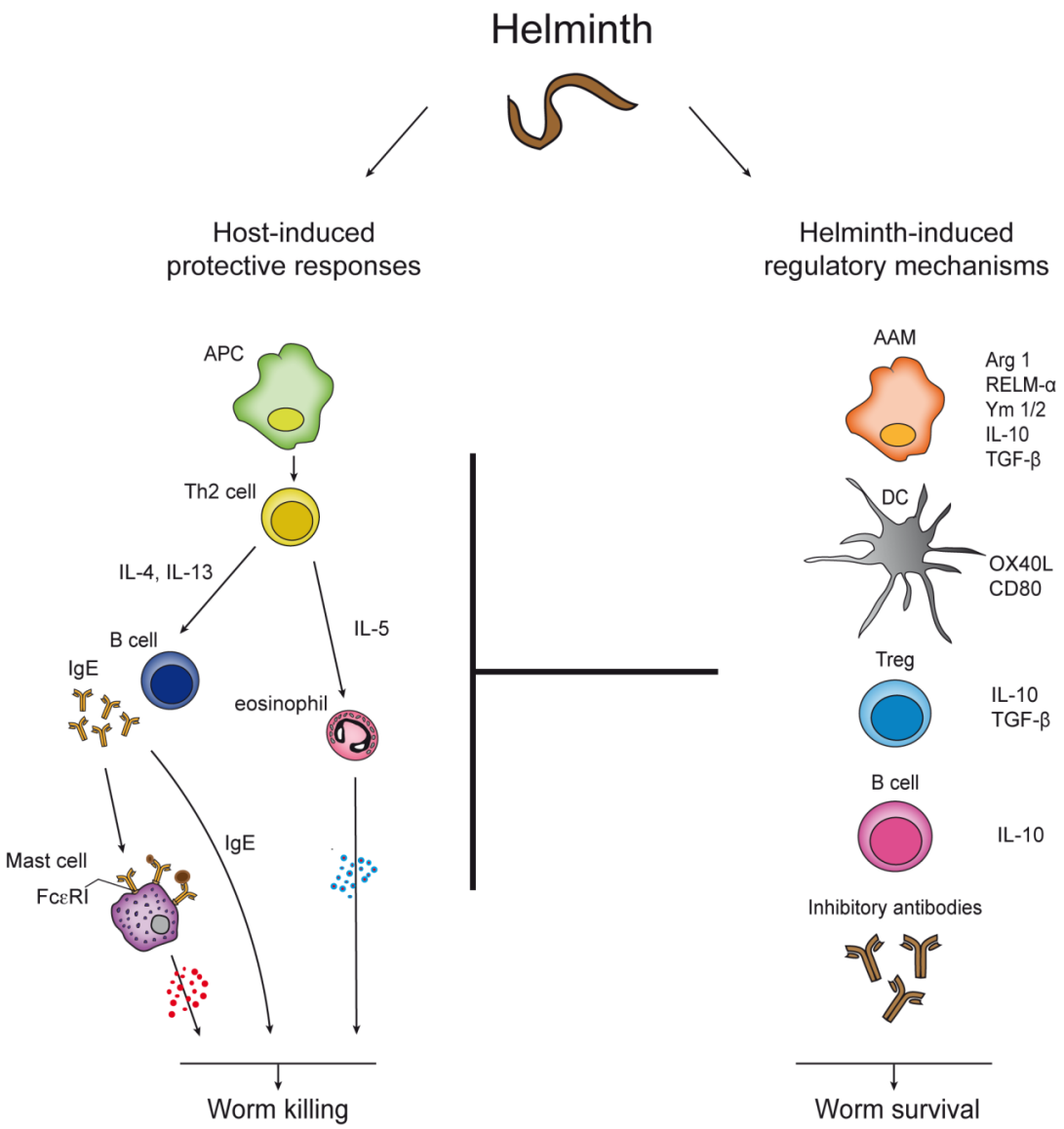


Figure 1-3. Protection and immune regulation in helminth infection.
Host-induced protective responses: Professional antigen presenting cells (APC) process helminth antigens and present them to $CD4^+$ T cells that differentiate into polarised Th2 cells. Th2 cells produce cytokines that activate and attract innate and adaptive cells. IL-4 and IL-13 induce differentiation of antigen-specific B cells and production of large amounts of antibodies, typically IgE. Antibodies opsonise helminths by binding to Fc receptors (R) and activating mast cells, eosinophils and neutrophils to kill the parasite by antibody-dependent cellular cytotoxicity. Sensitized mast cells secrete large amounts of histamine and other mediators and facilitate the attraction and accumulation of further immune cells, resulting in worm killing.
Helminth-induced regulatory mechanisms: Helminths target immune cells and induce the differentiation of alternatively activated macrophages (AAMs), dendritic cells (DCs), regulatory T cells (Treg), and B cells. AAMs in mice express arginase-1 (Arg 1), resistin-like molecule (RELM)-α, Ym-1, Ym-2, IL-10 and transforming growth factor (TGF)-β and contribute to wound healing. Tregs produce IL-10 and TGF-β, while B cells elicit regulatory mechanisms via expression of IL-10 and inhibitory antibodies. These cellular changes lead to a modified Th2 immune response and ultimately worm survival[50].

1.2.3.1. Host cells targeted by helminths to induce regulation

Immune modulation occurs through production of specific parasite-derived molecules that target mammalian host immune cells and signalling pathways. As mentioned, this depends on the presence of live parasites[48]. Furthermore this modulation can inhibit bystander responses: killing of schistosome parasites was shown to reinstate immune responses to a vaccine targeting human immunodeficiency virus-1, which were greatly diminished in the presence of the parasite[51]. Similarly, prior elimination of intestinal helminths in patients vaccinated with bacille Calmette-Guerin resulted in greater protective responses to the vaccine compared with patients who did not receive antihelminthic chemotherapy[52]. During active infection, live worms are thus essential to ensure continuous release of immunomodulatory molecules. This section describes the main host cells targeted by helminths, while details of specific immunomodulatory molecules are provided later.

Macrophages are clear targets of helminth-induced immune regulation. Macrophages that are activated by the Th2 cytokines IL-4 and IL-13 develop an alternatively activated phenotype and have a well-described role in helminth infections (reviewed elsewhere[53]). Alternatively activated macrophages are recruited in large numbers to the sites of infection where they can proliferate[31]. Alternatively activated macrophages are important in tissue homeostasis, downregulation of the adaptive immune system, acting as effector cells against parasites, and to reduce or heal any ensuing damage caused by infection[54]. Macrophages targeted in *B. malayi* infection induce a suboptimal antigen-specific T cell population whereby cell proliferation but not Th2 cell cytokine production is impaired[55]. This has been shown to be IL-4-dependent and requires direct contact with T cells to induce hyporesponsiveness, enabling parasite survival[56,57]. In gastrointestinal helminth infections, large numbers of alternatively activated macrophages accumulate in the gut. Macrophage depletion prevents smooth muscle hypercontractility in the small intestine and results in impaired worm expulsion[58].

The directed targeting of dendritic cells (DCs) represents another way in which helminths induce regulation. Toll-like receptor (TLR) responsiveness, expression of costimulatory molecules and production of pro-inflammatory cytokines in DCs are suppressed in helminth infection[59-64]. Helminth infection directs DCs to promote a modified Th2 response that instructs the $CD4^+$ T cell compartment[65,66]. Human DCs exposed to *B. malayi* microfilariae showed higher levels of apoptosis and decreased production of IL-12 and IL-10[67]. In fact when human monocytes that were being differentiated to DCs *in vitro* were stimulated with *B. malayi* microfilarial antigen, they produced significantly decreased levels of IL-12p40, IL-12p70 and IL-10 in response to bacterial adjuvant[62]. However, DCs that have been previously exposed to helminth products can also effectively prime Th2 cells[65], as is the case in schistosomiasis infection[68]. Thus the course of disease and the pathogen involved may produce different responses.

$CD4^+$ Tregs are known to reduce pathology in the host via suppression of both Th1 and Th2 responses (reviewed elsewhere[69]). In *Schistosoma mansoni* infection $CD4^+CD25^+$ T cells express high quantities of IL-10 and prevent pathology during chronic infection[70]. Furthermore this inhibits DC-derived IL-12, thus suppressing Th1 responses[71]. Both natural and adaptive Tregs have been described in filaria-infected persons, with the adaptive Treg population producing high levels of IL-10[72]. Similarly in *Heligmosomoides polygyrus* infection, $Foxp3^+$ Tregs are required to limit immunopathology and represent a potential source of IL-10[73,74]. During infection, Tregs are therefore important effector cells required to inhibit or reduce pathology in the host by modulating the ensuing Th2 response, thereby simultaneously allowing establishment of chronic infection.

Host protection and regulation by antibodies and B cells is recognised as an essential component of the Th2 response in helminth infections[75]. In a murine model of acute *S. mansoni* infection where the dominant isotypes are IgG1 and IgE[75], removal of B cells results in high levels of IFN-γ and IL-12 but low levels of IL-4 and IL-10[76]. Moreover mice deficient in B cells are unable to downregulate granuloma formation in chronic infection. This is mediated by the Fcγ receptor (R), which indicates a role for antibodies in down-modulation of pathology[77]. In fact, antibody isotypes have an important role in determining the outcome

of helminth infection in the host. The cytokines IL-4 and IL-13 act on B cells to induce both IgG1 and IgE in mice and IgG4 and IgE in humans. High levels of IgG4 but low levels of IgE are found in the blood of filaria-infected hyporesponsive, asymptomatic persons[78,79]. IgG4 is known to have downstream suppressive effects, including inhibiting complement activation[80]; thus IgG4 may prevent immunopathological responses in asymptomatically infected individuals.

1.2.3.2. Helminth-derived immunomodulatory molecules

In recent years research has focused on identifying defined immunomodulatory helminth-derived molecules that induce a microenvironment beneficial to the parasite. These functionally and structurally diverse molecules interact directly with host cells. A summary of helminth-derived immunomodulatory molecules is given in **Table 1-1.** Broadly, these molecules include the following:

(i) Parasite-derived homologues of host mammalian cytokines and chemokines. Two transforming growth factor (TGF)-β homologues found in Brugian species, *Bm-tgh-1* and *Bm-tgh-2*, have been well characterised, the second of which is thought to have an immunomodulatory role. TGH-2 is secreted by adult worms and in its recombinant form was shown to bind the human TGF-β receptor, thus potentially influencing Treg development[81,82]. *S. mansoni* male worms express a member of the TGF-β receptor family known as SmRK-1[83] for which mammalian TGF-β may be a ligand involved in worm development[84]. *H. polygyrus* excretory/secretory (ES) products contain remarkable TGF-β-like activity, inducing Foxp3 expression in naïve T cells and modulating immune functions, thereby maintaining worm burdens to induce chronic infection[85]. Macrophage migration inhibitory factor (MIF) homologues have been described in multiple helminth species (reviewed elsewhere[86,87]) that target human cells. Pastrana and colleagues described a MIF homologue in *B. malayi*, *W. bancrofti* and *O. volvulus*[88]. In fact, *B. malayi* MIF directly affected human monocyte behaviour by preventing random migration of cells[88]. While mammalian MIF has numerous functions, in particular as a pro-inflammatory cytokine[87], helminth MIF has direct chemotactic effects on human monocytes but appears to be associated with anti-inflammatory, modified Th2-type responses[89].

(ii) ES-62 is a secreted 62-kDa glycoprotein from *A. viteae* that exhibits a plethora of well-documented anti-inflammatory properties[90] and contains phosphorylcholine moieties, which are largely responsible for immunomodulation (reviewed elsewhere[91]). It was shown that ES-62 acts on macrophages to inhibit production of IL-12 if the cells were subsequently exposed to lipopolysaccharide (LPS) and IFN-γ[92,93]. ES-62 could also be shown to act on bone marrow-derived precursors of DCs to inhibit a pro-inflammatory response induced by LPS[94], and acted on B cells by modulating T and B cell interactions[95].

(iii) Schistosome soluble egg antigen (SEA) and ES products that contain potent Th2-inducing and immunomodulatory activity[96]. It was shown that omega-1, a hepatotoxic ribonuclease, is one of the key players in the SEA response[68,97]. Omega-1 is a glycoprotein that was demonstrated to polarise human monocyte-derived and $CD11c^+$ murine DCs in a direction supporting Th2 responses even in the presence of LPS. In fact SEA depleted of omega-1 was not able to sufficiently induce a Th2 response *in vitro*[97]. However, the Th2-suppressive actions of schistosome-derived antigens clearly highlight the ability of this helminth to modulate host immune responses. ES- or omega-1-treated DCs display the typical modulated phenotype that is critical for induction of a Th2 response, including reduced expression of costimulatory molecules and a lowered efficiency in participating in DC-T cell conjugates[68]. In fact this study demonstrated that omega-1 could alter the morphology of DCs, thereby possibly preventing T cell activation.

(iv) Cysteine and serine protease inhibitors that display potent immunomodulatory activity[98]. Of the helminth-derived immunomodulators, filarial cystatin, a well-characterised protease inhibitor of helminths, has high immunomodulatory potential. Filarial cystatin contributes to a large part of this thesis and will be discussed further in depth in the next section.

Table 1-1. Helminth-derived immunomodulatory molecules.

Helminth species	Name	Molecule	Mechanisms of immune modulation during infection	Ref.
Echinococcus granulosus	AgB	Antigen B	Reduces expression of costimulatory molecules on human DCs and induces Th2 responses.	99
Fasciola hepatica	FheCL1	Cysteine protease	Suppresses macrophage inflammatory mediators by degrading TLR3.	100
Schistosoma mansoni	Lyso-PS	Lyso-phosphatidylserine	Lyso-PS treated DCs induce IL-10$^+$ Treg through TLR2 and promote Th2 polarization.	66,101
	DsRNA	Double-stranded RNA	Triggers TLR3 to activate STAT1 and induces expression of type 1 IFNs in DCs.	102,103
	Omega-1	Ribonuclease	Reduces expression of costimulatory molecules and IL-12 in DCs and induces IL-4 and Foxp3 expression in CD4$^+$ T cells.	68,97,104
	IPSE/ alpha-1	Glycoprotein	Induces IgE-dependent IL-4 production from basophils *in vivo*.	105
	SmCKBP	Chemokine-binding protein	Suppresses neutrophil recruitment by inhibiting the mammalian chemokine CXCL8.	106
Ascaris suum	PAS-1	200 kDa protein	Suppresses pro-inflammatory cytokines and neutrophil influx after exposure to LPS.	107
Necator americanus	Na-ASP-2	High homology to C-C chemokines	Secreted by infective larvae, recruits neutrophils *in vitro* and *in vivo*.	108
	Na-NES	Metalloprotease	Cleaves the eosinophil chemoattractant CCL11 and prevents its action *in vitro* and *in vivo*.	109
Nippostrongylus brasiliensis	Acetylhydrolase	Acetylhydrolase	Inactivates mammalian platelet-activating factor, thus potentially regulating gastrointestinal inflammation.	110
Trichinella spiralis	Ts-MIF	MIF homologue	Inhibits migration of human PBMCs, similar to human MIF.	111
Trichuris muris	43kDa IFN-γ homologue	IFN-γ homologue	Binds to IFN-γ receptor in mice.	112
Acanthocheilonema viteae	AvCystatin/ Av17	Cysteine protease inhibitor	Down-regulates T cell responses. Interferes with macrophage MAP kinase signalling pathways to induce IL-10.	113,114
	ES-62	Phosphorylcholine-containing glycoprotein	Reduces CD4$^+$ T cell proliferation and IL-2 production, inhibits IL-4 and IL-13 production. Inhibits IL-12 production from macrophages after exposure to LPS in a TLR4-dependent manner. Induces an anti-inflammatory phenotype in DCs.	92-94,115
Brugia malayi	Bm-ALT-1, Bm-ALT-2	Abundant larval transcript proteins	Expression of *Bm*-ALT in recombinant *Leishmania mexicana* parasites diminishes IFN-γ mediated killing and induces GATA-3 and SOCS-1 in	116

			these macrophages.	
	Bm-CPI-2	Cysteine protease inhibitor	Blocks antigen presentation via MHC class II by interfering with asparaginyl endopeptidase.	[117,118]
	Bm-TGH-2	TGF-β homologue	Binds mammalian TGF-β receptors, may influence Treg differentiation.	[81]
Litomosoides sigmodontis	Ls-cystatin	Cysteine protease inhibitor	Reduces antigen-specific proliferation of spleen cells.	[119]
Onchocerca volvulus	Onchocystatin	Cysteine protease inhibitor	Suppresses proliferation of antigen-specific PBMCs, induces IL-10 from stimulated PBMCs. Reduces expression of MHC class II molecules and CD86 on human monocytes.	[120,121]

C(X)CL, chemokine ligand; DC, dendritic cell; ES, excretory/secretory products; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MHC, major histocompatibility complex; MIF, macrophage migration inhibitory factor; PBMC, peripheral blood mononuclear cell; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; Th, T helper cell; TLR, Toll-like receptor; Treg, T regulatory cell[50].

1.2.3.3. Filarial cystatin

Mammalian cysteine proteases are essential for efficient processing and presentation of antigen on major histocompatibility complex (MHC) class II to induce an appropriate adaptive T cell response. Mammalian cystatins play a vital role in regulating these pathways. However, filarial cystatins from *A. viteae*, *B. malayi*, *Nippostrongylus brasiliensis*, and *O. volvulus* have been shown to interfere with this process to dampen antigen-dependent immune responses (reviewed elsewhere[98]). Bm-CPI-2, a cystatin from *B. malayi*, was illustrated to interfere with antigen processing, which led to a reduced number of epitopes being presented to T cells *in vitro*[117]. Recombinant onchocystatin (rOv17) from *O. volvulus* was shown to reduce antigen-driven proliferation of PBMCs in a monocyte-dependent manner[121]. Recombinant AvCystatin (rAv17) from *A. viteae* has potent immunomodulatory roles illustrated by its ability to reduce antigen-specific and –unspecific T cell responses[113]. AvCystatin is recognised by macrophages and upon uptake induces phosphorylation of the mitogen-activated protein (MAP) kinase signalling pathways ERK1/2 and p38 in macrophages. This leads to tyrosine kinase-dependent IL-10 production in macrophages[113,114]. The rodent filarial species *L. sigmodontis* secretes a cystatin at various stages during its lifecycle, which after injection into the peritoneal cavity of *L. sigmodontis*-infected mice greatly decreases nitric oxide production and proliferation of antigen-specific spleen cells[119]. Similarly, recombinant cystatin from *N. brasiliensis* (named nippocystatin, NbCys) inhibits the cathepsins L and B, and suppresses antigen processing by antigen-presenting cells (APCs)[122]. Taken together, cystatins are a class of molecules found in numerous filarial species that have important immunomodulatory functions.

1.3. Biological functions of monocytes & macrophages

The above data mention throughout that, among other cells, monocytes and macrophages are targeted by helminths to induce immune modulation. The next section will describe the defining roles of monocytes and macrophages in health and disease and will highlight phenotypic and functional differences between human and murine cells. Finally an in-depth analysis is given of the vital roles of monocytes and macrophages in helminth infections.

1.3.1. Monocytes

Monocytes are innate cells that develop in the blood from common myeloid precursors in the bone marrow[123,124] and are characterised by expression of the LPS co-receptor CD14. They comprise over 5% of all leukocytes in the blood. Under steady state conditions, monocytes migrate from the blood into tissues within 2-3 days, where they differentiate into macrophages or other cell types, depending on the tissue microenvironment. During infection or inflammation monocytes are recruited within 8-12 hs, where they differentiate into macrophages of a particular phenotype, depending on the stimulus[125,126]. Nevertheless, before migration and differentiation to other cell types in the tissues, monocytes themselves have important roles in phagocytosis and cytokine production[127].

Research in the 1980s and 1990s demonstrated that human monocytes could be subdivided into distinct subsets based on their differential expression of the receptors CD14 and CD16 (also known as FcγRIII)[128,129]. Thereafter, nomenclature of the different subsets became rather unclear due to monocyte subset variation in different species and the immunological tools used to identify expressed receptors. Thus, defined nomenclature was proposed by a group of experts to describe the distinct subsets and published in 2010 by Ziegler-Heitbrock *et al.*[130]. From this review, human monocytes are categorised as belonging to one of three subsets. 'Classical' monocytes make up over 90% of the monocytes in the body and show high expression of CD14 (abbreviated as $CD14^{++}$) and do not express CD16 ($CD16^{-}$). The second subset is that of 'intermediate' monocytes, which have high expression of CD14 but intermediate expression of CD16 ($CD14^{++}CD16^{+}$). Finally, the third subset is that of 'non-classical' monocytes. In humans, monocytes of this subset are $CD14^{+}CD16^{++}$. Non-classical monocytes make up approximately 10% of all monocytes in the body[130]. The nomenclature arises from the observation that during infection, the numbers of 'intermediate' cells rise, followed by an increase in the numbers of 'non-classical' monocytes. Thus, there is believed to be a relationship between all three subsets, and that monocytes mature from one subset through to the others[130]. The subsets have each at some point in their existence been termed either inflammatory or anti-inflammatory[127,131,132]. Suffice to say that each subset may respond differently to a given stimulus under varying conditions, while the relationship among the subsets implies that the cells may acquire different phenotypes and functions throughout their maturation.

1.3.2. Macrophages

1.3.2.1. The murine system

A great deal of knowledge exists on murine macrophage subsets due to unlimited access to tissue macrophages. In the mouse, four distinct macrophage activation states have been described, which are dependent on the surrounding microenvironment (**Figure 1-4**)[123,133,134]. Classically activated (M1) macrophages are activated by a combination of LPS and IFN-γ[135] and in response to these stimuli will express inducible nitric oxide synthase (iNOS) and upregulate MHC II as well as the activation markers CD80 and CD86. Classically activated macrophages are particularly important in intracellular bacterial killing[136].

Macrophages in mice that are not classically activated are given the umbrella term 'M2' and have been subdivided further into the categories M2a, M2b and M2c. M2a macrophages are induced by IL-4[137] or IL-13[138] and represent the typical 'alternatively activated macrophage'. Ligation of FcRs combined with a TLR stimulus on activated macrophages induces the M2b activation state, also known as Type II activation[139,140]. The final murine subset, M2c,

includes a group of deactivated or regulatory macrophages, and develops in response to glucocorticoids, TGF-β or IL-10[134,141]. M2 macrophages are involved in tissue homeostasis, wound healing and downregulation of the adaptive immune system[54]. They act as effector cells against parasites and reduce or heal pathology caused during infection, occasionally causing fibrosis and the development of granulomas[142,143].

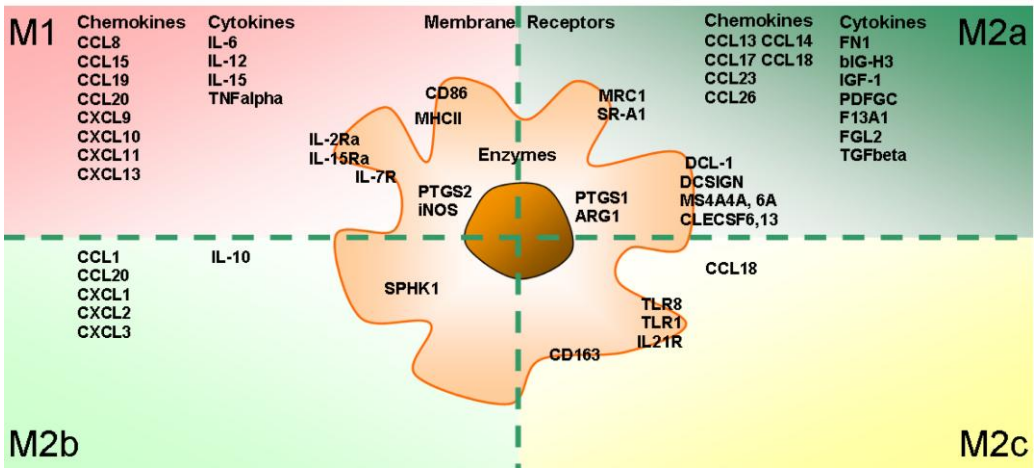


Figure 1-4. Macrophages can develop into one of four different activation states. The molecules expressed by each activation state are indicated in the figure. M1 or classically activated macrophages develop in response to stimulation with LPS plus IFN-γ. M2 macrophages are further subdivided. Thus, M2a or alternatively activated macrophages develop in response to stimulation with IL-4 and IL-13. M2b (also known as Type II activated) macrophages develop in response to stimulation with immune complexes plus a TLR ligand or an IL-1 receptor ligand. M2c or deactivated macrophages develop in response to stimulation with glucocorticoids, TGF-β or IL-10[134].

1.3.2.2. The human system

To obtain tissue macrophages from humans and to prove the existence of each of the above-described subsets *in vivo* is extremely difficult; nevertheless, a number of studies in different infection settings show the differentiation of human classically activated, alternatively activated or regulatory macrophages. Studies have attempted to differentiate human monocytes to macrophages *in vitro* that exhibit the same subdivided activation states as described for mice. One hurdle in translating findings from murine to human macrophages is the controversy in the field on what effector molecules and surface markers to use for human macrophage subset classification, as some key markers in the mouse are absent in the human system, difficult to detect or not unique to human macrophages[144].
Human classically activated macrophages can be activated by stimulation with GM-CSF[145], LPS[134] or IFN-γ *in vitro*, leading to the production of iNOS, hydrogen peroxide and killing of intracellular parasites[146]. In stark contrast to murine classically activated macrophages, human macrophages differentiated in response to pro-inflammatory stimuli produce extremely low or undetectable levels of NO[147]. However, in line with murine data, human classically activated macrophages express MHC II and CD86, and the inflammatory cytokines IL-12, tumour necrosis factor (TNF)-α and IL-6[148,149]. After activation, classically activated macrophages upregulate antigen presenting and phagocytic functions[150].
Soon after the discovery that IFN-γ induced classically activated macrophages in humans[146], IL-4 was shown to inhibit pro-inflammatory activity in human monocytes[151,152]. Similar to murine macrophages, human macrophages develop an alternatively activated phenotype when stimulated with IL-4 *in vitro* (reviewed elsewhere[153]). *In vivo*, human alternatively activated macrophages are distinguished by expression of the scavenger receptors CD163 and CD204, as well as the chemokine ligand CCL18 and the mannose receptor MRC-1 (CD206)[134,148,154]. Human tumour-associated macrophages have an M2-like phenotype and are important in regulating tumour development and directing the adaptive immune

response[155]. They express high levels of IL-10 but low levels of IL-12, and release factors that induce tumour growth, angiogenesis and tissue remodelling[156-158]. Intestinal macrophages also develop a distinct profile with similarities to M2 macrophages, as LPS stimulation of intestinal macrophages does not induce expression of the typical inflammatory cytokines IL-1, IL-6 or TNF-α[159]. Human monocytes have been shown to produce IL-10 in response to stimulation with glucocorticoids[160] and develop an anti-inflammatory phenotype[161,162] mirroring murine M2c macrophages. A role for human regulatory macrophages has recently been described to provide suppression of the immune response in solid organ transplants, thereby promoting tolerance[163]. Treatment of *in vitro* generated monocyte-derived macrophages with IFN-γ can help prevent organ rejection when administered to an allogeneic donor, by suppressing T cell proliferation[164].

1.3.3. The role of monocytes & macrophages in filarial infection

A role for macrophages in lymphatic filariasis was first determined in the 1980s when it was shown that macrophages become activated in murine models of filarial infection[165]. Subsequent research demonstrated the importance of macrophages in inducing a specific hyporesponsiveness in the T cell compartment[55]. Suppressive macrophages could inhibit T cell proliferation but not cytokine production in *B. malayi* experimentally infected mice, which was dependent on live parasites but independent of NO. It was suggested that these macrophages were responsible for the mechanism of immune suppression in asymptomatic, hyporesponsive individuals infected with the same species[55]. Down-regulation of macrophage activation was also demonstrated in a jird infection of *B. pahangi*; macrophages differentiated after exposure to adult worms did not become activated (as measured by TNF-α production) or produce NO after LPS stimulation[166]. The development of suppressive macrophages was dependent on IL-4 but not IL-5 or IL-10 from the host[167,168]. To inhibit T cell proliferation, cell-to-cell contact between macrophages and T cells was essential[57]. T cell-derived Th2 but not Th1 cytokine responses remained intact, and eventually it could be demonstrated that these macrophages induce a Th2 response[56]. Furthermore, this response is not restricted locally, enabling macrophages to regulate peripheral responses as well[169].

The macrophage population that develops in murine filarial infection has thus been well characterised and bears similarities to alternatively activated macrophages. In particular murine studies, this subset is also referred to as nematode-elicited macrophages, which display many properties in common with alternatively activated macrophages differentiated *in vitro* in response to IL-4. Nematode-elicited macrophages developed in response to adult worms typically express a combination of the markers arginase (arg)-1, resistin-like molecule (RELM)-α, Ym-1, Ym-2, acidic mammalian chitinase (AMCase), and MRC-1[133]. Macrophage-derived IL-10 contributes to worm patency, as over-expression of IL-10 in FVB mice (an otherwise resistant strain) resulted in complete susceptibility to infection[170]. Macrophage recruitment in filarial infection has also been elucidated in recent years. In murine infection, CCL2 was shown to be important for macrophage recruitment in acute intraperitoneal infection[171]. It was also recently shown that, under Th2 conditions, macrophages proliferate locally to increase cell numbers[31]. This highlights the role of IL-4 in both proliferation and differentiation of the macrophage subset, to ensure a hyporesponsive phenotype. Whether this also occurs in human infection remains to be determined[172].

PBMCs from filariasis asymptomatically infected patients that are stimulated with *B. malayi* adult antigen *in vitro* elicit monocytes that express arg-1, MRC-1, resistin, and CCL18[173]. Monocytes from *W. bancrofti* asymptomatically infected persons have reduced ability to produce IL-1β in response to LPS compared with monocytes from endemic normals[36]. In fact, during patent filarial infection monocytes encounter the microfilarial lifecycle stage in the blood, before migrating out to the tissues. This initial contact with the parasite is particularly interesting as it occurs only in asymptomatically infected patients where the adult worms are tolerated in the lymphatics and produce viable microfilariae[174]. Furthermore treatment of filaria-infected persons reverses monocyte dysfunction[175]. This suggests that microfilariae in active infection may induce a regulatory monocyte/macrophage phenotype in susceptible individuals that may in turn contribute to the outcome of infection.

1.4. Antibody glycosylation in lymphatic filariasis

An abundance of literature demonstrates the importance of antibody responses in filarial infection, involved in host protection as well as immune modulation of the host response. Additionally, outwith parasitic infections, there is growing evidence of a correlation between specific glycan structures in the Fc region of IgG and disease activity. Thus there is a potential role for differential glycan structures in the Fc region of IgG in the two clinical groups of persons exposed to lymphatic filariasis (see **Figure 1-2**) to regulate the clinical outcome.

1.4.1. Antibody structure

Antibodies are an integral component of the adaptive immune system that can eliminate pathogens and produce a longlasting memory response. They have vital functions in immunity to recognise and remove viruses, bacteria and parasites. Antibodies are secreted by plasma cells, and can exist as either a membrane-bound receptor attached to B cells (known as a B cell receptor) or as a secreted protein[176]. When a B cell encounters a pathogen and recognises cognate antigen, the B cell will become activated and develop into an antibody-producing plasma cell or a memory cell. In this way large amounts of specific antibody are produced that can neutralise the pathogen, activate complement, and directly activate other cells of the immune system via binding to Fc receptors (R).

Antibodies have a weight of approximately 150 kDa and have a Y-shaped structure. This structure consists of two heavy and two light chains. There are 5 different types of heavy chain represented by Greek letters that define the 5 classes of antibody: IgA (α), IgD (δ), IgE (ε), IgG (γ) and IgM (μ). Additionally each antibody contains one of two types of light chain, κ and λ. IgA exists as a dimer, while IgM exists as a pentamer; IgD, IgE and IgG exist as monomers. Antibodies consist of a variable region and a constant region. The variable region (Fab, fragment antigen-binding) contains a highly diverse amino acid sequence to allow for recognition of a vast number of antigens, while the constant region (Fc, fragment crystallisable) contains less diversity and is involved in FcRs binding on innate cells such as monocytes and macrophages and thus determines antibody effector functions[176]. Detailed functions of antibodies in filarial infections and of the Fc region in innate cell activation are discussed below.

1.4.2. The role of antibodies in lymphatic filariasis

Helminths specifically target various host responses to induce Th2-type and regulatory responses, of which antibody-derived host protection and regulation are essential components (reviewed in detail elsewhere[75,78]). In *W. bancrofti*, *B. malayi* and *O. volvulus* infection, there is a fine balance in the ratio of parasite-specific IgG4 to IgE, and different antibody subclasses are associated with the different clinical manifestations[177]. Asymptomatically infected persons carry high levels of antigen-specific IgG4[178]. In contrast, individuals with clinical disease have low levels of antigen-specific IgG4 but high IgE[24,179]. Literature on polyclonal antibody responses in lymphatic filariasis is scarce; however, a similar pattern of polyclonal IgG4 and IgE as described above has been reported by Ottesen *et al.*[178].

A great deal of literature exists on the role of these two isotypes, IgE and IgG4, in filarial nematode infections. Murine IgE has been shown to be active in killing microfilariae *in vitro*[180]. IgE can induce degranulation of mast cells, basophils and eosinophils and induce antibody-dependent cell-mediated cytoxicity[75]; in lymphatic filariasis it is thus suspected to be involved in parasite killing. In contrast, IgG4 may play a vital role in protection from disease and promotion of the asymptomatic response, particularly as IgG4 is induced in B cells by Tregs through IL-10 signalling[181] and can inhibit complement activation[80]. Thus, antibodies have an important role in helminth infections that may alter the outcome of disease.

1.4.3. Fc receptor-mediated activation of innate immune cells

The FcR on an innate immune cell recognises the Fc region of an antibody and can induce downstream signalling, resulting in inhibition or induction of an immune response. The FcR will recognise different antibodies depending on the class of antibody[182]. Furthermore, in mammals there are different classes of FcR that have varying properties. Thus, the Fc region of IgG can be recognised by FcγRI-IV; the class of FcR that is engaged will determine the outcome of the immune response. FcγRI, III and IV are activating receptors that engage cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs), leading to cytokine production and activation of other cells[183]. In contrast FcγRIIB is an inhibitory receptor that engages a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM). ITIM signalling suppresses ITAM activation and thereby suppresses immune responses[183].

FcγRI has the highest affinity of all the FcγRs for its ligand, and exhibits a relatively narrow specificity. In contrast FcγRII and FcγRIII have a broader specificity allowing them to bind a wider range of antibody isotypes. However, this comes at the cost of a much lower level of affinity for IgG Fc[182,184]. Finally FcγRIV has an intermediate affinity compared with the other receptors that shows restricted specificity[185].

Innate cells, including neutrophils, natural killer cells, mast cells, basophils, DCs, monocytes and macrophages use FcγRs to contribute to the immune response. Via FcγRs, neutrophils and natural killer cells recognise infected cells or pathogens that have become opsonised by IgG, and destroy them through antibody-dependent cell cytotoxicity[186]. FcεRs on mast cells and basophils recognise and become crosslinked by IgE inducing degranulation of these cells. Monocytes, macrophages and DCs, as key innate immune cells at the interface of innate and adaptive immunity, use FcRs to recognise pathogenic challenges and induce or suppress the ensuing immune response, depending on whether the FcR is activating or inhibitory[186]. Recognising opsonised antigen allows monocytes, macrophages and DCs to internalise antigen for degradation or processing and presentation on major histocompatibility complex (MHC) class II.

As described in **Figure 1-4,** macrophages develop into the M2b activation state when stimulated with immune complexes plus a TLR ligand[134]. These macrophages produce high levels of IL-10 but low levels of IL-12, in contrast to macrophages stimulated with only a TLR ligand such as LPS[187,188]. M2b macrophages may therefore protect the host by inhibiting IL-12 and downstream inflammation. Alternatively this mechanism can be employed by pathogens to subvert the immune response, as demonstrated in *Leishmania* spp, where an M1/Th1 response is associated with clearance of infection[189]. Opsonised *Leishmania* parasites have been shown to block expression of IL-12 and induce IL-10 expression from M2b macrophages through FcR crosslinking, a mechanism that is dependent on the presence of activating FcRs[190,191].

DCs rely on FcRs recognising opsonised antigen to become activated and mature, enabling efficient internalisation of antigen and presentation on MHC class II. Regnault *et al*. could show that antigen-antibody immune complexes induced greater DC maturation through upregulation of MHC class II, CD86 and CD40 compared with LPS alone[192]. Furthermore, this was dependent on the presence of the FcγR-associated γ chain required for ITAM activation[192]. Similarly, in a model of systemic lupus erythematosus, Means and colleagues demonstrated that FcγRII was required for plasmacytoid DCs to internalise immune complexes and induce cytokine production[193]. Thus in summary, FcRs play a vital role in immune activation, and the nature of the antibody-FcR engagement determines the outcome of the response.

1.4.4. Glycosylation

Glycosylation is defined as the attachment of covalently linked carbohydrates to a molecule such as a protein (known as a glycoprotein) or lipid (glycolipid)[194]. Glycosylation can occur either co-translationally or post-translationally. The glycosylation profile of different proteins or lipids varies greatly and can be used as a defining characteristic of particular molecules. Variation in glycan structure is introduced by addition of different monosaccharide structures in varying quantities. The main structures found in the human body are shown in **Figure 1-5**.

Glycans have important functions in many biological processes, including cell growth, adhesion and immune responses[194]. In general, glycans are involved in either modulating the structure and function of the molecules to which they are bound or in glycan recognition by carbohydrate-binding proteins (lectins). In vertebrates, approximately 50% of all proteins are glycosylated; glycoproteins, specifically antibodies, are the focus of this next chapter.
Glycans can be either *O*-linked or *N*-linked. *O*-linked glycans are attached to an oxygen atom of a serine or threonine residue in a protein. There is no defined amino acid sequence known for *O*-linked glycans[194]. *O*-linked glycans can be involved in blood clotting, embryogenesis, cell development and immunity.
N-linked glycans are glycans that are attached to a nitrogen atom of an asparagine (Asn, N) residue, with a minimal amino acid sequence required of asparagine-X-serine/threonine (where X represents any amino acid other than proline)[194]. *N*-linked glycans represent approximately 90% of all glycosylated proteins in humans, and play a vital role in protein structure and function. *N*-linked glycosylation occurs in the endoplasmic reticulum and Golgi complex, through the activity of glycosidases and glycosyltransferases[195].

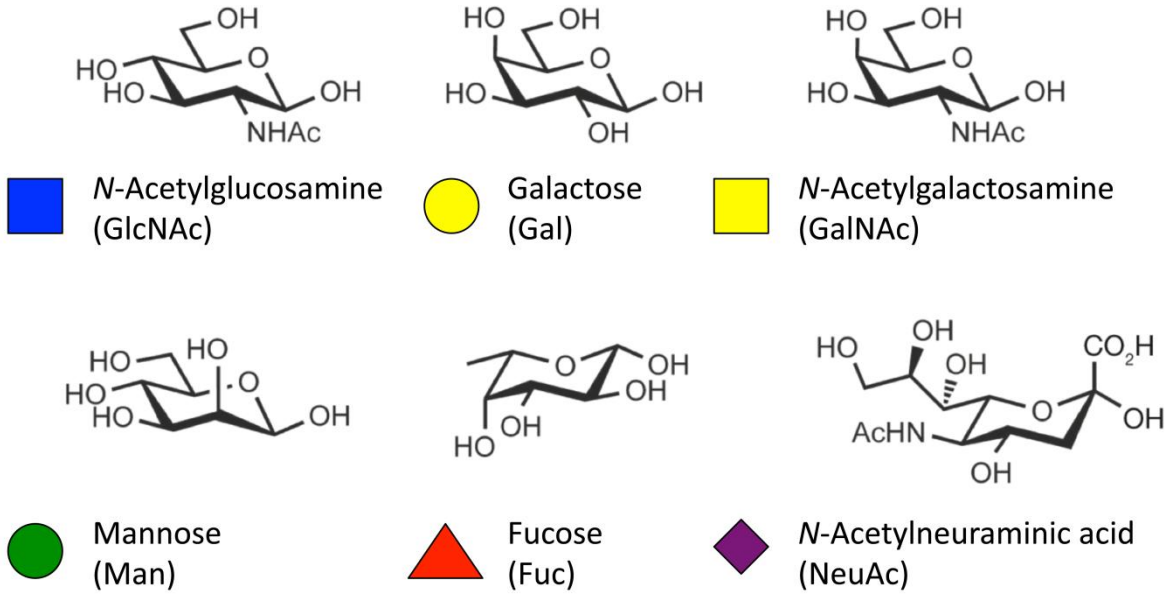


Figure 1-5. Representation of the main monosaccharide structures found in humans. Each structure is defined by a coloured symbol, applied uniformly throughout the literature. Adapted from elsewhere[194].

1.4.5. Antibody glycosylation

Both the Fc region and the Fab region of antibodies are glycosylated, a process that is essential for the structure and molecular activity of the antibody. In IgG, approximately 15-20% of Fab regions are glycosylated, in both the heavy and light chains. This occurs during somatic hypermutation, and is important for the improved recognition and binding of antigen. In contrast all Fc regions of IgG (in all subclasses) are glycosylated. A single *N*-linked glycan is found attached to the Asn-297 residue in each CH2 domain of both heavy chains (**Figure 1-6A**). It should be noted that other antibody isotypes (IgM, IgD, IgE and IgA) have different glycosylation properties; however, that topic is outwith the scope of this thesis[194].
The *N*-linked glycan structure in the Fc region has a core structure that is composed of *N*-acetylglucosamine and mannose (**Figure 1-6B**). Variation is produced by introducing bisecting *N*-acetylglucosamines, branching or terminal residues consisting of mannose, galactose and sialic acid and addition of fucose to the core.

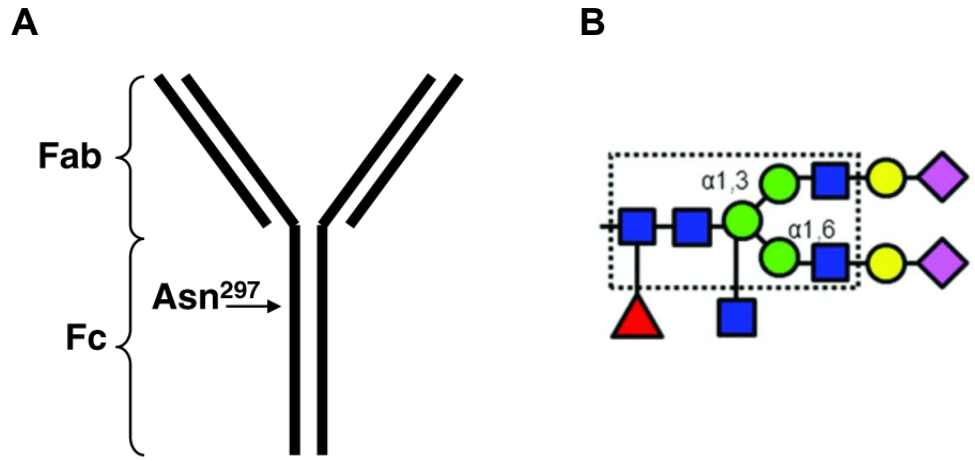


Figure 1-6. The structure of IgG and the Fc region *N*-linked glycan.
A) The Y-shaped structure of IgG, indicating the antigen binding region (Fab) and the Fc receptor-binding region. Asparagine (Asn)-297 marks the attachment site of the *N*-linked glycan. B) *N*-linked glycans attached to IgG have a core structure indicated by the rectangular dotted box that consists of *N*-acetylglucosamine (blue squares) and mannose (green circles). Variation is introduced through bisecting *N*-acetylglucosamine, branching or terminal residues of galactose (yellow circles) and sialic acid (purple diamonds), or addition of fucose (red triangle) to the core. The two mannose arms are designated α1,3 or α1,6 (adapted from elsewhere[196,197]).

The glycan in the Fc region ensures that the IgG molecule is correctly folded and maintains an open conformation of the two heavy chains, required for interaction with FcγRs[197,198]. The glycan composition regulates binding to different activating or inhibitory FcγRs, determining the response of effector cells carrying these receptors. This initiates various effector functions, including phagocytosis and killing of pathogens, cytokine production, expression of costimulatory molecules, engagement with adaptive immunity and activation of complement (reviewed in detail[197,199]). Thus, determining the glycan composition of IgG may give an indication of the nature of the immune response that develops and that may ultimately influence the clinical outcome of disease.

1.4.6. The role of IgG glycosylation in disease

Variation that is introduced by the Fc glycan allows for a diverse range of IgG antibodies with differing functions. The *N*-glycosylation profile of IgG thus has a major influence on inflammatory and regulatory responses in the host and can profoundly affect the outcome of disease[200]. Altered IgG glycosylation profiles are known to correlate with disease, whereby decreased galactosylation is typically associated with inflammation or cancer[201]. In rheumatoid arthritis (RA), disease onset is associated with serum agalactosylated IgG[202], while treatment with anti-TNF results in increased galactosylation[203]. In patients with RA who become pregnant, galactosylation increases and correlates with a remission in disease[204]. In a murine model of the skin disorder epidermolysis bullosa acquisita it could be shown that galactosylation of IgG1 was essential to maintain anti-inflammatory responses in an FcγRIIB- and dectin-1-dependent manner[205].
Alongside changes in galactosylation, increased sialylation typically correlates with anti-inflammatory responses. One classic example of this is the anti-inflammatory potential of intravenous immunoglobulin (IVIG, used to treat many autoimmune diseases) that is a result of sialylation in the IgG Fc region[206]. It could be shown that sialic acid and the inhibitory receptor FcγRIIB were essential for the anti-inflammatory activity of IVIG[206-208]. Furthermore, increases in IgG sialylation are associated with improvements in RA during pregnancy[204]. To conclude, sialic acid in the Fc region of IgG shows a strong correlation with anti-inflammatory functions, which acts through FcγRIIB to reduce disease.

2. AIMS OF THE STUDY

2.1. Open questions & the contribution of this thesis

It is clear that helminths have multiple methods to manipulate the immune system and induce a microenvironment that allows their lifecycle to be maintained in the relative absence of disease. Helminth-derived immunomodulatory molecules that target specific host cells are crucial in driving this regulation. Additionally, a great deal is known about monocytes and macrophages in disease and the different roles that these cells can adopt, both in filarial and unrelated infections. Nevertheless, a defined role for monocytes and macrophages in regulating innate and adaptive responses in human filarial infection has not been unequivocally demonstrated. Furthermore, the phenotype of these cells in persons who characterise the different clinical outcomes in lymphatic filariasis is not known. The potential of filarial cystatin to regulate disease outcome through human monocyte/macrophage modulation is unclear and deserves further investigation. Finally, a role for antibody glycosylation in the development of filarial infection and disease has until now not been researched. This thesis addresses these issues and thereby to contribute to the understanding of immune regulation and clinical outcome in human filariasis.

2.2. Hypothesis

First, I hypothesise that microfilariae specifically modulate human monocytes and macrophages *in vivo* in patent lymphatic filarial infection. This results in a distinct phenotype that interferes with innate and adaptive immune responses. This phenotype contributes to the development of asymptomatic infection, and may be partly induced by filarial cystatin.
Second, I hypothesise that the *N*-linked glycosylation profile of IgG contributes to the development of the spectrum of responses seen in lymphatic filariasis. This may influence the development of infection through the engagement of different FcγRs, resulting in activation or inhibition of different effector cells.

2.3. Aims and objectives

The aims of this thesis are:

(i) To establish monocytes and macrophages as targets of immune modulation by *B. malayi* female and microfilarial (Mf) lysate *in vitro*.

(ii) To determine the contribution of filarial cystatin to the immune modulation of monocytes and macrophages *in vitro*.

To complete the above two aims, I will characterise the phenotype and function of monocytes and macrophages from filaria non-endemic normal donors stimulated *in vitro* with *B. malayi* female lysate, Mf lysate and filarial cystatin.

(iii) To establish monocytes as targets of immune modulation by microfilariae *in vivo* during active infection.

To complete this aim, I will characterise the phenotype of monocytes that develop *in vivo* in filaria-exposed donors who comprise the two described clinical groups (asymptomatic infection and chronic pathology) and compare them with monocytes isolated from endemic normals.

(iv) To determine the role of the IgG glycosylation profile in the development of lymphatic filarial infection.

To complete this aim, I will compare the *N*-linked glycosylation profile in the Fc region of total IgG in plasma from filaria-exposed donors who comprise the two described clinical groups (asymptomatic infection and chronic pathology) and compare the results with those from endemic normals.

3. MATERIALS

3.1. Biological resources

B. malayi microfilariae and adults	FR3, Athens, Georgia, USA
Buffy coats	German Red Cross, Dresden, Germany
Eschericia coli LPS K12 strain	Invivogen, California, USA
Recombinant Bm-CPI-2	Prepared in house
Recombinant AvCystatin	Prepared in house

3.2. Laboratory equipment

ABI 7300 Real-Time PCR	Life Technologies, California, USA
AutoMACS classic	Miltenyi Biotec, Bergisch-Gladbach, Germany
Beckman P/ACE MDQ System	Beckman-Coulter, Brea, California, USA
Casy cell counter, model TT System	Innovatis, Roche, Mannheim, Germany
Cell incubator	ThermoFisher Scientific, Schwerte, Germany
Centrifuges	Eppendorf, Hamburg, Germany
FACS Canto II	Becton Dickinson, New Jersey, USA
Hydrospeed microplate washer	Tecan, Männedorf, Switzerland
Mastercycler Nexus	Eppendorf, Hamburg, Germany
NanoDrop ND-1000	Peqlab Biotechnologie, Erlangen, Germany
Synergy HT plate reader	BioTek, Vermont, USA
Ultrasonicator	ThermoFisher Scientific, Schwerte, Germany
Vacuum centrifuge, Univapo 150 ECH	Uniequip, Planegg, Germany
Horizontal shaker & incubation hub	Unimax 1000 & 1010, Kelheim, Germany

3.3. Consumables

6- and 24-well cell culture plates	Corning Costar, Bodenheim, Germany
96 flat and 96 round cell culture plates	Corning Costar, Bodenheim, Germany
96 round maxisorp NUNC-immuno plates	Nunc, Wiesbaden, Germany
96-well multiply-PCR plates	Sarstedt, Nümbrecht, Germany
Alltech C18 Extract-Clean cartridges	ThermoFisher Scientific, Schwerte, Germany
Amicon centrifugal filters	Merck, Darmstadt, Germany
C-Chip disposable hemocytometer	Digital Bio NanoEnTek, Seoul, Korea
Eppendorf tubes (1.5 and 2 ml)	Eppendorf, Hamburg, Germany
Falcon cellstar tubes (15 and 50 ml)	Greiner bio-one, Frickenhausen, Germany
Filter tips	Greiner bio-one, Frickenhausen, Germany
MACS MS columns	Miltenyi Biotec, Bergisch-Gladbach, Germany
MACS pre-separation filter (70 µm)	Partek, St. Louis, USA
PCR soft tubes (0.2 ml)	Biozym, Hessisch Oldendorf, Germany
Sealing tape for 96-well PCR plate	Sarstedt, Nümbrecht, Germany
Single use filter (0.22 µm)	Sartorius Stedim Biotech, Goettingen, Germany
Stripette (10, 25 and 50 ml)	Corning Costar, Bodenheim, Germany
Transfer pipettes (3.5 ml)	Sarstedt, Nümbrecht, Germany
Vivacell 70 concentrators (5000 MW)	Sartorius Stedim Biotech, Göttingen, Germany

3.4. Buffers and media

3.4.1. *Brugia malayi* purification and culture

Parasite purification medium	RPMI-1640 200 U/ml penicillin 200 mg/ml streptomycin
Parasite culture medium	RPMI-1640 1% glucose 200 U/ml penicillin 200 mg/ml streptomycin

3.4.2. Cell culture and preparation

PBMC wash	PBS 0.2% (v/v) BSA
Erythrocyte lysis buffer (pH 7.5)	0.01 M $KHCO_3$ 0.155 M NH_4Cl 0.1 mM EDTA
Complete RPMI	RPMI-1640 5% human AB serum 100 U/ml penicillin, 100 mg/ml streptomycin 1 mM L-glutamine 1 mM MEM non-essential amino acids 1 mM sodium pyruvate
Monocyte/macrophage harvest	PBS 5 mM EDTA
Phagocytosis uptake buffer (pH 7.4)	140 mM NaCl 2.5 mM KCl 1.8 mM $CaCl_2$ 1.0 mM $MgCl_2$ 20 mM HEPES

3.4.3. Cell sorting and flow cytometry

MACS running buffer	PBS 0.2% BSA 2 mM EDTA
MACS rinse buffer	PBS 2 mM EDTA
Flow cytometry buffer	PBS 0.2% BSA 2 mM EDTA

3.4.4. ELISA

Carbonate coating buffer (pH 9.5)	0.1 M $NaHCO_3$ 0.095 M Na_2CO_3
Wash buffer	PBS 0.05% Tween20

Blocking buffer	PBS 3% BSA
Stopping solution	1 M H_2SO_4

3.4.5. Antibody glycan analysis

IgG elution buffer (pH 3.0)	0.1 M glycine/HCl
IgG neutralisation buffer (pH 8.1)	1 M Tris/HCl
Pepsin inactivation buffer (pH 9.0)	1 M Tris/HCl

3.5. Chemicals and biological reagents

3.5.1. Chemicals

Bovine serum albumin (BSA)	AppliChem, Darmstadt, Germany
EDTA	AppliChem, Darmstadt, Germany
Ethanol (absolute)	AppliChem, Darmstadt, Germany
Glucose	Merck, Darmstadt, Germany
Human serum, type AB, “off the clot”	Merck, Darmstadt, Germany
L-glutamine	PAN Biotech, Aidenbach, Germany
Lymphocyte Separation Medium (LSM)	PAN Biotech, Aidenbach, Germany
MEM non-essential amino acids	PAN Biotech, Aidenbach, Germany
Paraformaldehyde	Carl Roth, Karlsruhe, Germany
Penicillin	PAN Biotech, Aidenbach, Germany
Phosphate-buffered saline (PBS)	PAN Biotech, Aidenbach, Germany
Phosphate-citrate buffer tablets	Sigma-Aldrich, Steinheim, Germany
RPMI-1640	PAN Biotech, Aidenbach, Germany
Sodium pyruvate	PAN Biotech, Aidenbach, Germany
Streptomycin	PAN Biotech, Aidenbach, Germany
Tetramethyl benzidine chromogen (TMB)	Sigma-Aldrich, Steinheim, Germany
Trifluoroacetic acid	Merck, Darmstadt, Germany
Tween20	Carl Roth, Karlsruhe, Germany
Water (molecular biology grade)	AppliChem, Darmstadt, Germany

3.5.2. Microbeads and antibodies

Anti-human CD14 microbeads	Miltenyi Biotec, Bergisch-Gladbach, Germany
Anti-human CD14-PE-Cy5 (clone RMO52)	Beckman Coulter, Florida, USA
Anti-human HLA-DR-APC (clone G46-6)	Becton Dickinson, New Jersey, USA
Anti-human CD11b-FITC (clone M1/70)	eBioscience, California, USA
Anti-human CD80-PE (clone 2D10.4)	eBioscience, California, USA
Anti-human CD86-PE (clone IT2.2)	eBioscience, California, USA
Anti-human CD163-PE (clone GHI/61)	eBioscience, California, USA
Anti-human CD273-PE (clone MIH18)	eBioscience, California, USA
Anti-human CD274-PE (clone MIH1)	eBioscience, California, USA
Anti-human CD4-PE-Cy5 (clone RPA-T4)	BioLegend, California, USA
Anti-human CD3 (clone OKT3)	eBioscience, California, USA
Anti-human IL-10 (clone JES3-9D7)	eBioscience, California, USA
Anti-human PD-1 (clone J116)	eBioscience, California, USA
Anti-human IgG1 (clone 4E3)	Acris Antibodies, Herford, Germany
Anti-human IgG2 (clone 31-7-4)	Acris Antibodies, Herford, Germany
Anti-human IgG3 (clone HP6050)	Acris Antibodies, Herford, Germany
Anti-human IgG4 (clone HP6025)	GeneTex, California, USA
Anti-human IgE	Bethyl Laboratories, Inc., Texas, USA
Anti-human IgG4	Santa Cruz Biotechnology, Texas, USA
FcR Blocking Reagent	Miltenyi Biotec, Bergisch-Gladbach, Germany
Fixable Viability Dye eFluor 780	eBioscience, California, USA

3.5.3. Cytokines

Human M-CSF	Peprotech, New Jersey, USA
Human IL-4	Peprotech, New Jersey, USA
Human IFN-γ	Peprotech, New Jersey, USA

3.5.4. Other reagents

APTS	Sigma-Aldrich, Steinheim, Germany
CFSE	Sigma-Aldrich, Steinheim, Germany
Cytochalasin D	Gibco, Life Technologies, California, USA
DNA-ExitusPlus	AppliChem, Darmstadt, Germany
Maltose	Sigma-Aldrich, Steinheim, Germany
N-glycosidase F 100 mU	Roche Diagnostics, Rotkreuz, Switzerland
Protein A ceramic hyperD F beads	Pall Life Sciences, Dreieich, Germany
Pepsin	Sigma-Aldrich, Steinheim, Germany
RNase-ExitusPlus	AppliChem, Darmstadt, Germany
Sequencing grade modified trypsin	Promega, Madison, USA

3.6. Commercial kits

BCA protein assay kit	ThermoFisher Scientific, Schwerte, Germany
$CD4^+$ T cell Isolation Kit II	Miltenyi Biotec, Bergisch-Gladbach, Germany
FastStart Universal SYBR Green	Roche Applied Science, Indianapolis, USA
High capacity RNA-to-cDNA kit	Life Technologies, California, USA
Human IgG1 Ready-Set-Go	eBioscience, California, USA
Human IgG2 Ready-Set-Go	eBioscience, California, USA
Human IgG3 Ready-Set-Go	eBioscience, California, USA
Human IgG4 Ready-Set-Go	eBioscience, California, USA
Human IL-4 ELISA Ready-Set-Go	eBioscience, California, USA
Human IL-6 ELISA Ready-Set-Go	eBioscience, California, USA
Human IL-8 ELISA Ready-Set-Go	eBioscience, California, USA
Human IL-10 ELISA Ready-Set-Go	eBioscience, California, USA
Human IL-13 ELISA Ready-Set-Go	eBioscience, California, USA
Human IL-27 ELISA Ready-Set-Go	eBioscience, California, USA
Human IFN-γ ELISA Ready-Set-Go	eBioscience, California, USA
Human TNF-α ELISA Ready-Set-Go	eBioscience, California, USA
Human IL-12/23 p40 ELISA MAX	BioLegend, California, USA
InnuPREP RNA mini-kit	Analytik Jena, Jena, Germany
Limulus amoebocyte lysate QCL 1000	Lonza, Basel, Swtizerland
pHrodo BioParticles	Life Technologies, California, USA
TropBio Og4C3 ELISA	TropBio Pty. Ltd, Australia

3.7. Primer sequences

Primer pairs were synthesized by and purchased from TIB-MOLBIOL (Berlin, Germany) or www.RealTimePrimers.com (Pennsylvania, USA). Accession numbers from NCBI database.

Table 3-1. Primer pair sequences used for RT-PCR.

Target	Accession number		Sequence
β 2-microglobulin	NM_004048	Forward	5'-TGC TGT CTC CAT GTT TGA TGT ATC T-3'
		Reverse	5'-TCT CTG CTC CCC ACC TCT AAG T-3'
CCL2	NM_002982	Forward	5'-AGT GTC CCA AAG AAG CTG TG-3'
		Reverse	5'-GAT TCT TGG GTT GTG GAG TG-3'
CCL18	NM_002988	Forward	5'-TGT GCT GAC CCC AAT AAG AA-3'
		Reverse	5'-GGC ATA GCCA GAT GGG ACT CT-3'
IL-6	NM_000600	Forward	5'-ATG CAA TAA CCA CCC CTG AC-3'
		Reverse	5'-GAG GTG CCC ATG CTA CAT TT-3'
IL-8	NM_000584	Forward	5'-TAG CAA AAT TGA GGC CAA GG-3'
		Reverse	5'-AGC AGA CTA GGG TTG CCA GA-3'
IL-10	NM_000572	Forward	5'-AAG CCT GAC CAC GCT TTC TA-3'
		Reverse	5'-ATG AAG TGG TTG GGG AAT GA-3'
IL-12p40	NM_002187	Forward	5'-TCC ATC AGG ATC AGT CCC TA-3'
		Reverse	5'-GGT TTG CAT TGT CAG GTT TC-3'
MRC-1	NM_002438	Forward	5'-GGC GGT GAC CTC ACA AGT AT-3'
		Reverse	5'-ACG AAG CCA TTT GGT AAA CG-3'
PD-L1	NM_014143	Forward	5'-TGA TAC ACA TTT GGA GGA GAC G-3'
		Reverse	5'-CCC TCA GGC ATT TGA AAG TAT C-3'
PD-L2	NM_025239	Forward	5'-AGG CCT TTG ATA ATT GGC ACT A-3'
		Reverse	5'-CCC AAA TTT TGC TCA GTT AAG G-3'
TNF-α	NM_000594	Forward	5'-TCC TTC AGA CAC CCT CAA CC-3'
		Reverse	5'-AGG CCC CAG TTT GAA TTC TT-3'

CCL, chemokine ligand; IL, interleukin; MRC, mannose receptor C; PD-L, programmed death-ligand; TNF, tumour necrosis factor.

3.8. Software

32-Karat software	Beckman-Coulter, California, USA
ABI 7300 SDS Software	Life Technologies, California, USA
FlowJo, version 8.8.7	Tree Star, Oregon, USA
GraphPad Prism version 6.0d	GraphPad Software, Inc., California, USA

4. METHODS

4.1. Characterisation of the phenotype and function of monocytes and macrophages from filaria non-endemic normal donors

4.1.1. Ethical statement

All experiments with material from filaria non-endemic normal donors were approved by the ethical committee of the Charité, Berlin (permit number EA1/104/14). The study was performed according to the Declaration of Helsinki.

4.1.2. *Brugia malayi* adult male, female and Mf lysate and ES preparation

Live *B. malayi* microfilariae and adult male or female worms were a kind donation from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3, Athens, Georgia). Microfilariae and adult male or female worms were washed twice in parasite purification medium (RPMI containing 200 U/ml penicillin and 200 µg/ml streptomycin) by centrifuging for 10 min at 450 g at 4°C. To collect excretory/secretory (ES) products, microfilariae were cultured for 3-5 days in parasite culture medium (RPMI containing 1% glucose, 200 U/ml penicillin and 200 µg/ml streptomycin) in a 5% CO_2-incubator at 37°C in 6-well cell culture plates whereby media was replaced every 24 hs. The resulting ES-containing media was concentrated using Vivacell 70 concentrators with a membrane cut-off of 5,000 MW. To prepare lysate, adult males and females or live microfilariae in suspension were subsequently harvested and washed twice in phosphate buffered saline (PBS) by centrifuging for 10 min at 450 g at 4°C. The adult worms or pelleted microfilariae were homogenised directly in a glass homogeniser and ultrasonicated on ice at an intensity of 10% for 3 min. The homogenate was centrifuged at 10,000 g and 4°C for 10 min and sterile filtered through a 0.22 µm filter. Protein concentration was determined using the Pierce BCA protein assay kit as per the manufacturer's guidelines. LPS concentration was determined by *Limulus* amoebocyte lysate (LAL) endotoxin detection kit; the LPS content in *B. malayi* microfilarial (Mf) lysate, male lysate, female lysate or ES used in all assays was < 1 EU/ml in the final concentration.

4.1.3. Isolation of PBMCs from buffy coats

PBMCs were isolated from buffy coats by density centrifugation using Lymphocyte Separation Medium (LSM). Buffy coats (80-100 ml) were initially diluted in PBS (two parts blood to one part PBS). Briefly, diluted buffy coats were layered onto LSM and centrifuged at 1260 g, at room temperature for 25 min with no brake and no accelerator. The interphase was collected and washed in PBS plus 0.2% bovine serum albumin (BSA). Cells were centrifuged at 450 g, 4°C for 10 min, washed in PBS plus 0.2% BSA and centrifuged at 200 g, 4°C for 10 min to remove platelets. Lysis of erythrocytes was performed to remove remaining erythrocytes if necessary. For this, 5 ml of erythrocyte lysis buffer were added to cells for 5 min at room temperature, after which cells were washed in PBS plus 0.2% BSA and centrifuged at 450 g for 10 min.

4.1.4. Isolation of $CD14^+$ monocytes from PBMCs and subsequent differentiation to macrophages

To positively select for $CD14^+$ monocytes, 200 µl of anti-CD14 beads were added to pelleted PBMCs from one buffy coat and incubated at 4°C for 20 min. Cells were washed in PBS plus 0.2% BSA, 2 mM EDTA and filtered before separation using a 70 µm filter. Cells were separated by positive selection, using an autoMACS classic and the program 'possel'. CD14 isolation was checked for purity by staining the cells with anti-CD14-PE-Cy5 (clone RMO52, 1:100), after which cells were acquired using the FACS Canto II and analysed using FlowJo, version 8.8.7. MACS isolation produced a $CD14^+$ cell purity of > 95%.

After isolation, cells were washed once in PBS plus 0.2% BSA, 2 mM EDTA, centrifuged at 450 g and 4°C for 5 min and transferred into complete RPMI medium (RPMI 1640, 5% AB human serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM L-glutamine, 1 mM MEM non-essential amino acids, 1 mM sodium pyruvate). Cells were counted using a Casy cell counter. Macrophages were generated *in vitro* by culturing $CD14^+$ monocytes in complete RPMI plus 10 ng/ml M-CSF in 6-well cell culture plates at a cell concentration of 0.33 x 10^6/ml and a density of 0.1 x $10^6/cm^2$ for 6 days, at 37°C and 5% CO_2. For macrophages differentiated in the presence of Mf lysate, 20 µg/ml *B. malayi* Mf lysate was added to the supernatant from the beginning of culture.

4.1.5. *In vitro* stimulation of monocytes and macrophages

Monocytes were seeded at a concentration of 2 x 10^6 per well into 24-well cell culture plates and stimulated *in vitro* with 1 ml stimulus. *In vitro* generated macrophages were used on day 6 of culture, whereby the culture supernatant was replaced with 1 ml stimulus.
To determine the response of monocytes and macrophages to known polarising agents, cells were stimulated for 4 hs and 24 hs with 20 ng/ml IL-4 or 100 ng/ml LPS plus 20 ng/ml IFN-γ, at 37°C and 5% CO_2.
To establish the optimal dose of *B. malayi* lysate to use, monocytes and macrophages were stimulated for 24 hs with 20 µg/ml, 10 µg/ml, 5 µg/ml or 2.5 µg/ml of *B. malayi* female or Mf lysate. For all other experiments, monocytes and monocyte-derived macrophages were stimulated *in vitro* with 20 µg/ml lysate for 4 hs or 24 hs at 37°C and 5% CO_2.
To determine the response of monocytes and macrophages to filarial cystatin, cells were stimulated with 20 µg/ml recombinant cystatin from *B. malayi* (rBm-CPI-2, expressed in an *Eschericia coli* expression system, prepared in house using a non-denaturing protocol). The concentration of 20 µg/ml was determined by titration, using IL-10 protein production as a readout. Thus, 20 µg/ml rBm-CPI-2 resulted in the highest level of IL-10 from monocytes as measured by ELISA (data not shown). LPS concentration was determined using a LAL endotoxin detection kit; the LPS content of rBm-CPI-2 was < 1 EU/ml in the final concentration.
After 24 hs stimulation, the supernatant was collected and stored at -20°C. After 4 hs or 24 hs, cells were washed in PBS. For this, the supernatant was removed and 1 ml PBS was added per well. Then the cells were washed by resuspension using a pipette. This was repeated three times. Finally, to measure gene expression by RT-PCR, RNA was lysed by addition of 400 µl RNA lysis buffer (part of the InnuPREP RNA mini-kit) directly into the well and stored at -80°C until further use.

4.1.6. Cytokine analysis

IL-6, IL-8, IL-10, IL-13, IL-27, IFN-γ and TNF-α protein were measured using commercial ELISA kits from eBioscience. IL-12p40 was measured using a commercial ELISA kit from BioLegend. All samples were measured in duplicates. Absorbance was read at 450 nm with background wavelength subtracted at 570 nm.

4.1.7. RNA extraction and real-time PCR

RNA was isolated from cells using the InnuPREP RNA mini-kit, following the manufacturer's instructions. The concentration of extracted RNA was determined using the NanoDrop 1000. RNA was reverse-transcribed to cDNA using a high capacity RNA-to-cDNA kit, following the manufacturer's instructions. cDNA was set to a concentration of 3-10 ng/µl, depending on the experiment. Real-time (RT) PCR was performed with FastStart Universal SYBR Green Master Mix using the ABI 7300 Real-Time PCR. For each sample, an end volume of 20 µl was used for RT-PCR, containing the SYBR Green Master Mix (10 µl), primers (1.2 µl for each primer, forward and reverse), water (6.6 µl) and cDNA (1 µl). For all genes, the primers were used at a concentration of 5 µM. For all genes, the following conditions were used, whereby the target was detected during the amplification stage:

Denaturation: 1 cycle, 95°C for 10 min
Amplification: 40 cycles, 95°C for 15 sec, 60°C for 30 sec, 72°C for 20 sec
Melting: 1 cycle, 95°C for 10 sec, 65°C for 1 min
Cooling: 1 cycle, 40°C for 10 sec

Relative changes in gene expression were calculated using the ABI 7300 SDS Software. Expression levels of transcripts were normalized to the Ct values of the endogenous housekeeping gene, ß2-microglobulin by using the $2^{-\Delta\Delta ct}$ method[209]. Relative expression of genes in stimulated samples was compared with unstimulated controls.

4.1.8. Harvesting of adhered monocytes and macrophages from culture plates

To remove non-adherent cells, monocytes or macrophages were washed in PBS. For this, the culture medium was removed and 1 ml PBS was added per well. Then the cells were washed by resuspension using a pipette. This was repeated three times, and the wash was discarded. To remove adhered monocytes and macrophages from the cell culture plate, 1 ml PBS containing 5 mM EDTA was added to each well for 10 min at 4°C. Cells were scraped off the well using a cell scraper, and centrifuged at 450 g for 10 min at 4°C. Cells were then centrifuged at 450 g and 4°C for 10 min and resuspended in complete RPMI and counted using a Casy cell counter.

4.1.9. Flow cytometry analysis of monocytes and macrophages

Monocytes, macrophages and macrophages differentiated in the presence of Mf lysate were harvested as in 4.1.8. and analysed for surface expression of HLA-DR, CD80, CD86, programmed death-ligand (PD-L)1, PD-L2, CD11b and CD163. Cells were treated with FcR Blocking Reagent (1:200) and stained with Fixable Viability Dye eFluor 780 (1:1000) and one or combinations of the following: anti-CD80-PE (clone 2D10.4, 1:100), anti-CD86-PE (clone IT2.2, 1:400), anti-CD274-PE (clone MIH1, 1:100), anti-CD273-PE (clone MIH18, 1:100), anti-CD11b-FITC (clone M1/70, 1:300), anti-CD163-PE (clone GHI/61, 1:50) or HLA-DR-APC (clone G46-6, 1:20). Cells were acquired using the FACS Canto II and analysed using FlowJo, version 8.8.7.

4.1.10. LPS stimulation of Mf lysate-differentiated macrophages

To determine the ability of Mf lysate-differentiated macrophages to respond to LPS stimulation, Mf lysate-differentiated macrophages were washed on day 6 of culture and 0.2 x 10^6 cells were stimulated in a 96-well round bottom plate for 24 hs with 100 ng/ml LPS. The following day supernatants were collected for cytokine analysis by ELISA.

4.1.11. Phagocytosis assay

Macrophages were generated *in vitro* and used after 6 days of incubation (see 4.1.4). Macrophages were harvested as in 4.1.8. Macrophages were seeded into a 96-well flat bottom plate (0.2 x 10^6 cells per well) in complete RPMI, and stimulated using 20 µg/ml *B. malayi* female or Mf lysate for 24 hs. After 24 hs macrophages were washed in PBS 3 times, by adding 200 µl PBS and carefully removing using a pipette. Then the prepared fluorescent pHrodo BioParticles suspension was added. pHrodo BioParticles were prepared beforehand by suspending 2 mg BioParticles in 2 ml of uptake buffer. The solution was briefly vortexed and then sonicated for 5 min to ensure homogenous dispersal of the particles. 100 µl of the prepared suspension was then added to the cells. Following the manufacturer's instructions, unstimulated macrophages were regarded as a positive control. Cytochalasin D (20 µM) was added to unstimulated cells as a negative control. Cells were incubated with the fluorescent particles for 3 hs at 37°C (no CO_2). The cells were washed three times in PBS, by adding 200 µl PBS and carefully removing the PBS using a pipette. Finally, 200 µl of 0.5% formalin was added. Fluorescence was read at 550 nm excitation and 600 nm emission. The net phagocytosis was calculated as per the manufacturer's instructions, by subtracting the average fluorescence intensity of the negative control from the positive control and all experimental wells. The phagocytosis response to the experimental effector (%

phagocytosis) could then be calculated as a percentage of the net positive control phagocytosis (% phagocytosis = net phagocytosis x 100 / net phagocytosis of positive control).

4.1.12. Isolation of $CD4^+$ T cells from PBMCs and CFSE labelling

PBMCs were labelled using the $CD4^+$ T cell Isolation Kit II according to the manufacturer's instructions and sorted by negative selection on an autoMACS classic using the program 'deplete'. Hereby, all non-$CD4^+$ T cells are magnetically labelled and removed from the sample. $CD4^+$ T cells were then washed twice in PBS (centrifuged at 450 g for 10 min at 4°C) and stained with CFSE. For this, the supernatant was replaced with 1 ml of 10 µM CFSE in 1 ml PBS. Cells were resuspended and stored at room temperature in darkness for exactly 8 min. To stop the reaction, 10 ml complete RPMI was added. $CD4^+$ T cells were then washed twice in 10 ml complete RPMI (centrifuged at 450 g for 10 min at 4°C) and rested in complete RPMI for 24 hs at 37°C and 5% CO_2.

4.1.13. Monocyte:$CD4^+$ T cell coculture

Monocytes were left unstimulated or stimulated with 20 µg/ml *B. malayi* Mf lysate or 20 µg/ml *B. malayi* microfilarial ES for 24 hs in a 5% CO_2-incubator at 37°C in 6-well cell culture plates. Monocytes were washed as in 4.1.5 and harvested as in 4.1.8. 0.1 x 10^6 monocytes were cocultured with 0.5 x 10^6 CFSE-labelled $CD4^+$ T cells in 96-well flat cell culture plates in the presence of 2 µg/ml soluble anti-CD3. For neutralization of PD-1 or IL-10, anti-PD-1 (clone J116) or anti-IL-10 (clone JES3-9D7) antibodies were added at 10 µg/ml at the beginning of culture. After 3-5 days, the supernatant was removed for cytokine analysis and cells were stained with Fixable Viability Dye eFluor 780 (1:1000) and anti-CD4-PE-Cy5 (1:100, clone RPA-T4). Cells were fixed in 200 µl of 0.5% formalin, acquired using the FACS Canto II and analysed using FlowJo, version 8.8.7.

4.2. Characterisation of the *Wuchereria bancrofti*-exposed cohort and determination of the phenotype and function of isolated monocytes

4.2.1. Ethical statement

All experiments with material from *W. bancrofti*-exposed donors were approved by the ethical committee of the Blue Peter Public Health and Research Center-LEPRA Society, Hyderabad (permit number 5/2009). Informed written consent was obtained from all participants. All *W. bancrofti* infected donors were treated for lymphatic filariasis by administration of DEC and symptomatic relief after completion of the study. The study was performed according to the Declaration of Helsinki.

4.2.2. Study population

Experiments using samples from filaria-exposed donors examined a cohort of 56 individuals from Andhra Pradesh in South India, where lymphatic filariasis caused by *W. bancrofti* is endemic. Night blood smears were performed with 20 µl blood to detect circulating microfilariae, and the TropBio Og4C3 ELISA was performed using plasma to detect circulating filarial antigen (CFA), as per the manufacturer's instructions. Patients with lymphatic filarial pathology (lymphadenitis, lymphoedema, hydrocoele) and with a history of lymphatic pathology (assessed by questionnaire) were examined as part of a clinical protocol approved by the institutional ethical committee of the Blue Peter Public Health and Research Center-LEPRA Society. Diagnosis was performed by a qualified medical doctor with experience in lymphatic filariasis. Based on these results, 28 individuals were classed as endemic normals (EN), 21 had chronic pathology (CP) and 7 had asymptomatic infection (AS) (**Table 4-1)**. Any AS patient found to be positive for CFA was classed as asymptomatic regardless of the night blood smear result; at 20 µl blood per smear, the test has low sensitivity, giving a cut off value of 50 microfilariae per ml.

Table 4-1. Diagnostic characteristics of the study cohort in Andhra Pradesh, South India, used to characterise monocyte phenotype.

	Number (M/F)	Median age (range)	Lymphatic pathology	microfilaria status	CFA status
Endemic normal	28 (15/13)	41 (15-63)	no	negative	negative
Chronic pathology	21 (4/17)	55 (34-74)	yes	negative	negative
Asymptomatic infection	7 (6/1)	33 (28-66)	no	negative/ positive	positive

M, male; F, female; CFA, circulating filarial antigen.

4.2.3. Measurement of polyclonal antibody isotypes in plasma

Plasma was isolated from 40 ml blood from filaria-exposed donors by density centrifugation using LSM. Blood was layered onto LSM and centrifuged at 1260 g at room temperature for 25 min with no brake and no accelerator, after which the plasma was removed and stored at -20°C until further use. Total IgE, IgG1, IgG2, IgG3 and IgG4 were measured using a commercial ELISA kit. Absorbance was read at 450 nm with background wavelength subtracted at 570 nm. A plasma titration was performed for each subclass to determine the optimal dilution to use for maximum resolution. Thus, plasma was diluted as follows for each subclass: IgE to 1:10; IgG1 to 1:2000; IgG2 to 1:500,000 or 1:1000,000; IgG3 to 1:40,000; IgG4 to 1:1000.

4.2.4. Measurement of *B. malayi* lysate-specific antibody isotypes in plasma

Plasma from *W. bancrofti*-exposed donors was tested for the presence of filarial-specific antibodies by testing for cross-reactivity to *B. malayi* mixed adult lysate (produced from adult males and females following the protocol described in 4.1.2 above). Plasma was isolated as in 4.2.3. Filarial-specific antibody isotypes (IgG1, IgG2, IgG3, IgG4 and IgE) were measured by ELISA. For IgG1-4, 96-well round bottom plates were coated overnight at 4°C with 2 µg/ml *B. malayi* lysate in carbonate buffer. The following day, plates were washed 3 times in PBS plus 0.05% Tween20. After each subsequent incubation or labelling step, plates were washed 3 times. Plates were blocked for 1 h at room temperature with PBS plus 3% BSA. A plasma titration (plasma diluted in PBS plus 3% BSA) was performed for each antibody subclass to determine the optimal dilution to use for maximum resolution: IgG1 to 1:500; IgG2 to 1:100; IgG3 to 1:100; IgG4 to 1:100. Plasma was added for 2 hs at room temperature. All samples were measured in duplicates. The presence of antibody was detected by addition of a secondary horse radish-peroxidase conjugated antibody diluted in PBS plus 3% BSA as follows: mouse anti-human IgG1 to 1:1000 (clone 4E3); mouse anti-human IgG2 to 1:1000 (clone 31-7-4); mouse anti-human IgG3 to 1:500 (clone HP6050): mouse anti-human IgG4 to 1:25,600 (clone HP6025). The secondary antibody was added for 1 h at room temperature. Substrate was detected by addition of tetramethyl benzidine solution for 20 min at room temperature in darkness. The reaction was stopped by adding 1 M H_2SO_4, and plates were read at 450/630 nm.

To measure *B. malayi* lysate-specific IgE, plasma samples were first depleted of IgG4, to avoid competition of IgG4 for the binding site shared by the two isotypes. Thus 96-well round bottom plates were coated overnight at 4°C with 2 µg/ml mouse anti-human IgG4 diluted in carbonate buffer. The following day plates were washed 3 times in PBS plus 0.05% Tween20. Undiluted plasma was added overnight at 4°C. The same day new plates were coated overnight at 4°C with 2 µg/ml *B. malayi* lysate in carbonate buffer. The following day plates were washed 3 times, and the same protocol as described above for *B. malayi* lysate-specific IgG was performed, with the addition of the IgG4-depleted plasma, undiluted. The presence of antibody was detected by adding an alkaline-phosphatase conjugated secondary goat anti-human IgE, diluted in PBS plus 3% BSA to 1:1000. Substrate was detected by adding p-nitrophenylphosphate solution for 30 min at 37°C. The reaction was stopped by adding 100 mM EDTA, and absorbance was read at 405/630 nm.

4.2.5. Measurement of filarial cystatin-specific antibody isotypes in plasma

Plasma from *W. bancrofti*-exposed donors was analysed for the presence of filarial cystatin-specific antibodies by testing for cross-reactivity to recombinant cystatin from *Acanthocheilonema viteae* (rAvCystatin). rAvCystatin was produced by expression in an *E. coli* expression system, prepared in house, according to an established protocol[210]. Plasma was isolated as in 4.2.3. rAvCystatin-specific antibody isotypes (IgG1, IgG2, IgG3, IgG4, and IgE) were measured by ELISA, as described in 4.2.4. Plates were coated using 2 µg/ml rAvCystatin in carbonate buffer. A plasma titration (plasma diluted in PBS plus 3% BSA) was performed for each antibody subclass to determine the optimal dilution to use for maximum resolution: IgG1 to 1:500; IgG2 to 1:100; IgG3 to 1:100; IgG4 undiluted; IgE undiluted.

4.2.6. Isolation of PBMCs from whole blood and subsequent purification of $CD14^+$ monocytes

To isolate PBMCs from 40 ml whole blood, the blood was layered onto LSM and centrifuged at 1260 g, at room temperature for 25 min with no brake and no accelerator. The interphase was collected and washed in PBS plus 0.2% BSA. Cells were centrifuged at 450 g, 4°C for 10 min, washed in PBS plus 0.2% BSA and centrifuged at 200 g, 4°C for 10 min to remove platelets. Lysis of erythrocytes was performed to remove remaining erythrocytes if necessary. For this, 5 ml of erythrocyte lysis buffer was added to cells for 5 min at room temperature, after which cells were washed in PBS plus 0.2% BSA and centrifuged at 450 g for 10 min. To positively select for $CD14^+$ monocytes, 50 µl anti-CD14 beads were added for

20 min to pelleted PBMCs and $CD14^+$ monocytes separated by positive selection using MACS MS columns as per the manufacturer's instructions.
After isolation, monocytes were washed once in PBS plus 0.2% BSA, 2 mM EDTA, centrifuged at 450 g and 4°C for 5 min and transferred into complete RPMI. Monocytes were counted using a C-Chip disposable hemocytometer. Monocytes were either stored in RNA lysis buffer for *ex vivo* RT-PCR analysis (as in 4.1.7) or seeded at a concentration of 0.1 x 10^6 per well into 96-well flat cell culture plates for *in vitro* stimulation.

4.2.7. RNA extraction and real-time PCR

RNA was isolated and RT-PCR was performed as per 4.1.7. For monocytes from filarial-exposed donors the data was not normalised to the reference group (endemic normals, EN), as there was a large variation in Ct values within this heterogeneous EN population. Thus, expression levels of transcripts were normalized to the Ct values of the endogenous housekeeping gene by using the method $½^{\Delta Ct}$ where ΔCt represents the difference between the target gene and the housekeeping gene. Baseline expression of samples from AS or CP was compared with that of EN. In all experiments ß2-microglobulin was used as a housekeeping gene.

4.2.8. *In vitro* stimulation of monocytes from *W. bancrofti* endemic donors

After seeding at a concentration of 0.1 x 10^6 per well into 96-well flat cell culture plates, monocytes were stimulated *in vitro*. For this, the culture medium was removed and replaced with 200 µl stimulus for 24 hs at 37°C and 5% CO_2. Cells were either left unstimulated or stimulated with 20 µg/ml *B. malayi* female lysate or Mf lysate. After 24 hs stimulation, the supernatant was collected and stored at -20°C until further analysis.

4.2.9. Cytokine measurement in the culture supernatant

IL-10, IL-6, TNF-α and IL-12p40 protein were measured in the culture supernatant of stimulated cells as in 4.1.6.

4.3. Analysis of IgG Fc *N*-linked glycosylation in *W. bancrofti*-exposed donors

4.3.1. Study population

Experiments on the glycosylation profile of filaria-exposed donors examined a cohort of 58 individuals from Andhra Pradesh in South India, where lymphatic filariasis caused by *W. bancrofti* is endemic. Individuals were diagnosed and classified as EN, CP or AS using the exact same methods as described previously (see 4.2.2.). Based on these results, 25 individuals were classed as endemic normals (EN), 25 had chronic pathology (CP) and 8 had asymptomatic infection (AS) (**Table 4-2**). The EN and CP groups were identified based on having a matching age range to the AS group, as age has been demonstrated to affect antibody glycosylation[211,212]. Additionally the number of persons studied for this experiment differs from that described in 4.2.2 as more plasma samples were collected from donors than cell samples. All experiments on the glycosylation profile of filaria-exposed donors were ethically approved as described previously (see 4.2.1.).

Table 4-2. Diagnostic characteristics of the study cohort in Andhra Pradesh, South India, used to characterize the glycosylation profile.

	Number (M/F)	Median age (range)	Lymphatic pathology	microfilaria status	CFA status
Endemic normal	25 (14/11)	46 (26-63)	no	negative	negative
Chronic pathology	25 (9/16)	50 (29-65)	yes	negative	negative
Asymptomatic infection	8 (6/2)	33 (28-66)	no	negative/ positive	positive

M, male; F, female; CFA, circulating filarial antigen.

4.3.2. Measurement of polyconal and filarial-specific antibody isotypes

Polyclonal and filarial-specific IgG1, IgG2, IgG3, IgG4 and IgE were measured in the study cohort following the same protocol as described above, in 4.2.3 and 4.2.4, respectively.

4.3.3. Purification of total IgG from plasma

To analyse the glycan profile of total IgG, the following method was employed, as established in house and published elsewhere[201]. This technique is regularly used to analyse the glycan profile of Fc region IgG[213-215]. To purify total IgG (IgG1, IgG2 and IgG4), 10 µl of plasma from each donor of the filaria-exposed cohort was incubated with 50 µl Protein A ceramic hyperD F beads in 600 µl PBS for 1 h at room temperature, rotating the sample continuously. The sample was allowed to settle, the supernatant was discarded, and the beads were transferred to a 10 µl filter tip. The column was washed with 3 x 50 µl PBS. Purified IgG was eluted using 0.1 M glycine, 3 M HCl, pH 3.0 and immediately neutralised using 1 M Tris, 3 M HCl, pH 8.1. The eluted sample was transferred to an Amicon 3 kDa centrifugal filter, and buffer exchanged into 0.1 M sodium acetate, pH 4.0 (14,000 g, 15 min).

4.3.4. IgG pepsin digestion and generation of Fc glycopeptides

To obtain the IgG Fc region, the purified IgG was digested with pepsin (2% w/w) overnight at 37°C in a horizontal shaker. Digestion was inactivated using 1 M Tris-HCl buffer, pH 9. The Fc fragments were purified by ultrafiltration using Amicon 10 kDa centrifugal filters (14,000 g, 15 min). The Fc region was recovered in the flow-through and dried in a vacuum centrifuge.

4.3.5. Enzymatic *N*-glycan release

Fc fragments, dissolved in 100 µl of 0.2 M PBS, pH 6.5 were digested with trypsin (1% w/w) overnight at 37°C in a horizontal shaker. After inactivating trypsin activity by incubation at 95°C for 5 min, *N*-glycosidase F 100 mU was added and the sample was incubated overnight at 37°C in a horizontal shaker.

4.3.6. Isolation and purification of released *N*-glycans

To remove contaminating peptides or glycoproteins, C18 Extract-Clean cartridges (Extract Clean SPE C18 100 mg/1.5 ml, Alltech) were washed in 80% acetonitrile (ACN) + 0.1% trifluoroacetic acid (TFA) and equilibrated in 0.1% TFA in water. The sample was adjusted to a pH < 4.0 by adding 10% TFA. The sample was applied to the C18 column, and glycans, collected in the flow-through, were concentrated by centrifugal evaporation. They were subsequently desalted using self-made graphite tips[201]. Graphite microcolumns were washed in 80% ACN + 0.1% TFA and equilibrated in 0.1% TFA. The sample was redissolved in 0.1% TFA and applied to the graphite tip, centrifuging briefly. The column was washed in 0.1% TFA. *N*-glycans were eluted in 25% ACN + 0.1% TFA. 100 pmol maltose was added as an internal standard. Samples were concentrated in a vacuum centrifuge.

4.3.7. APTS labelling of glycans

The glycans were labelled using 9-aminopyrene-1,3,6-trisulfonic acid (APTS) in 15% v/v acetic acid, 1 M sodium cyanoborohydride in THF, overnight in darkness at 37°C in a horizontal shaker. Glycans were purified over graphite columns (see 4.3.6), and the samples were redissolved in water.

4.3.8. CE-LIF *N*-glycan profiling

APTS-labelled *N*-glycans were analysed on a Beckman P/ACE MDQ System equipped with laser-induced fluorescence (LIF) (λ_{ex} = 488 nm, λ_{em} = 520 $\pm$ 10 nm) using the 32-Karat software. The background electrolyte consisted of 25 mM acetate buffer (pH 4.75) containing 0.4% polyethylene oxide. Surface area was calculated for each *N*-glycan structure by the 32-Karat software and given as a percentage of the total surface area of all structures in one sample.

4.4. Statistical analyses

All statistical analyses were performed using GraphPad Prism version 6.0d. In experiments where two paired groups were analysed, Wilcoxon signed-rank test was used to compare a condition to its unstimulated control. In experiments where more than two paired groups were analysed, Friedman's ANOVA was used to determine whether a statistically significant difference existed between any of the conditions and the unstimulated control (significance level $p<0.05$). In case of significance, the main analysis was followed up with a Wilcoxon signed-rank test between a condition and the unstimulated control, whereby a Bonferroni correction was applied. The Kruskal-Wallis test with Dunn's multiple comparisons post-test was used to determine statistical significance between multiple unpaired groups.

5. RESULTS

5.1. Characterisation of the phenotype and function of monocytes and *in vitro* generated macrophages from filaria non-endemic normal donors

5.1.1. Monocytes and macrophages respond appropriately to *in vitro* stimulation with known polarising agents

To determine the role of monocytes and macrophages in filarial disease it was first essential to show that these cells respond appropriately to stimulation with known polarising agents, as previously reported. Monocytes from healthy donors were stimulated or differentiated to macrophages. Monocytes or macrophages were stimulated for 4 hs or 24 hs with a classical activation stimulus, LPS (100 ng/ml) plus IFN-γ (20 ng/ml) or an alternative activation stimulus, IL-4 (20 ng/ml). Cytokine production was measured by ELISA, gene expression of selected markers was determined by RT-PCR and expression of activation and surface markers was determined by flow cytometry (**Figure 5-1**). Specific known markers for classically or alternatively activated monocytes/macrophages were examined[133].

Monocytes stimulated for 24 hs with LPS + IFN-γ produced significantly higher levels of IL-10, IL-6, TNF-α, IL-12p40 and IL-8, compared with unstimulated controls (**Figure 5-1A**). In contrast, IL-4 did not induce production of IL-10 or IL-12p40, while IL-6, TNF-α, and IL-8 were significantly downregulated. IL-27 was not detected in any conditions.

To assess the expression in monocytes of markers associated with an alternative or regulatory phenotype, mRNA expression of *MRC-1*, *CCL18*, *CCL2, PD-L1* and *PD-L2* was analysed after 4 hs and 24 hs stimulation (**Figure 5-1B**). Expression of cells stimulated with IL-4 or LPS + IFN-γ was normalised to unstimulated controls. Monocytes significantly upregulated expression of *CCL18*, *PD-L1* and *PD-L2* at 4 hs and 24 hs post stimulation with LPS + IFN-γ compared with unstimulated controls. Expression of *MRC-1* was significantly downregulated at 4 hs and 24 hs post stimulation with LPS + IFN-γ compared with unstimulated controls, while *CCL2* expression did not change. IL-4 induced significantly high expression of *MRC-1* at 4 hs and *CCL18*, *PD-L1* and *PD-L2* at 4 hs and 24 hs compared with controls. In contrast, *CCL2* was significantly downregulated at 24 hs.

To determine whether the high mRNA level of *PD-L1* was reflected on a protein level, surface expression of PD-L1 was measured by flow cytometry after 24 hs stimulation. Expression of the activation markers HLA-DR, CD80 and CD86 as well as PD-L2 was measured in parallel (**Figure 5-1C**). LPS + IFN-γ induced expression of HLA-DR, PD-L1 and PD-L2, while stimulation with IL-4 upregulated CD86, PD-L1 and PD-L2. CD80 was not altered after stimulation with LPS + IFN-γ or IL-4.

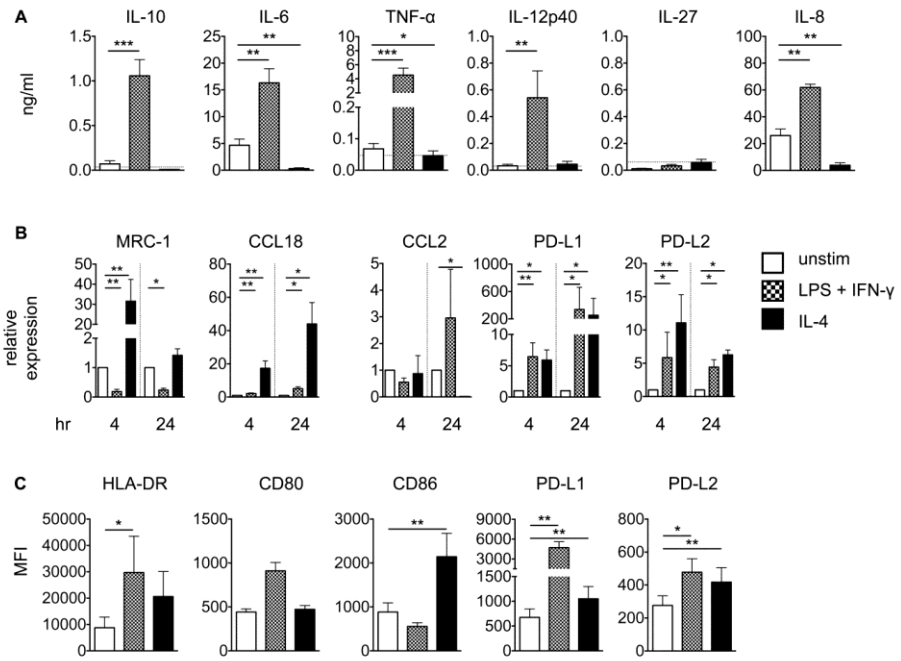


Figure 5-1. Monocytes respond appropriately to known polarising stimuli.
Monocytes from filaria non-endemic normal donors were left unstimulated or stimulated for 4 hs and 24 hs with LPS + IFN-γ or IL-4. A) Cytokine production was measured at 24 hs using ELISA. Results show pooled data from 4-8 experiments (n = 12-24). Horizontal dashed line indicates the limit of detection of the assay. B) mRNA expression was determined at 4 hs and 24 hs using RT-PCR. Results show pooled data from 3 experiments (n = 8-9). C) Surface expression of defined markers was measured at 24 hs by flow cytometry. Pooled data from 2-4 experiments (n = 6-10) are represented as the mean fluorescence intensity (MFI). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. * p<0.025, ** p<0.005, *** p<0.0005.

Similar to monocytes, *in vitro* generated macrophages produced significantly elevated protein levels of IL-10, IL-6, TNF-α, IL-12p40 and IL-8 after 24 hs stimulation with LPS + IFN-γ, while in contrast to monocytes, IL-27 was also detected (**Figure 5-2A**). IL-4 induced production of IL-27, while IL-10, IL-6, TNF-α, IL-12p40 and IL-8 remained unaltered.
On mRNA level, macrophages stimulated with LPS + IFN-γ expressed significantly higher levels of *PD-L2* at 4 hs and 24 hs post stimulation, while *CCL18* and *PD-L1* showed only transiently increased expression at 4 hs, compared with unstimulated controls (**Figure 5-2B**). *MRC-1* and *CCL2* were not changed compared with controls after stimulation with LPS + IFN-γ. IL-4 induced significant and high levels of *MRC-1* and *PD-L2* at 4 hs and 24 hs post stimulation of macrophages, whereas *CCL18* and *PD-L1* showed transient expression at 4 hs compared with unstimulated controls. *CCL2* remained unchanged compared with controls.
Surface expression of the markers HLA-DR, CD80, CD86, PD-L1 and PD-L2 was measured by flow cytometry after 24 hs (**Figure 5-2C**). Stimulation with LPS + IFN-γ increased expression of CD80, CD86, PD-L1 and PD-L2 compared with unstimulated controls; however, this did not reach significant levels. IL-4 also did not alter expression of these markers compared with unstimulated controls.
In summary, there are clear differences between human monocytes and macrophages in the kinetics and volume of expression of specific genes in response to known polarising stimuli. Nevertheless, both cell types responded as expected from published literature for human monocytes and macrophages stimulated with classical or alternative polarising stimuli[133,146].

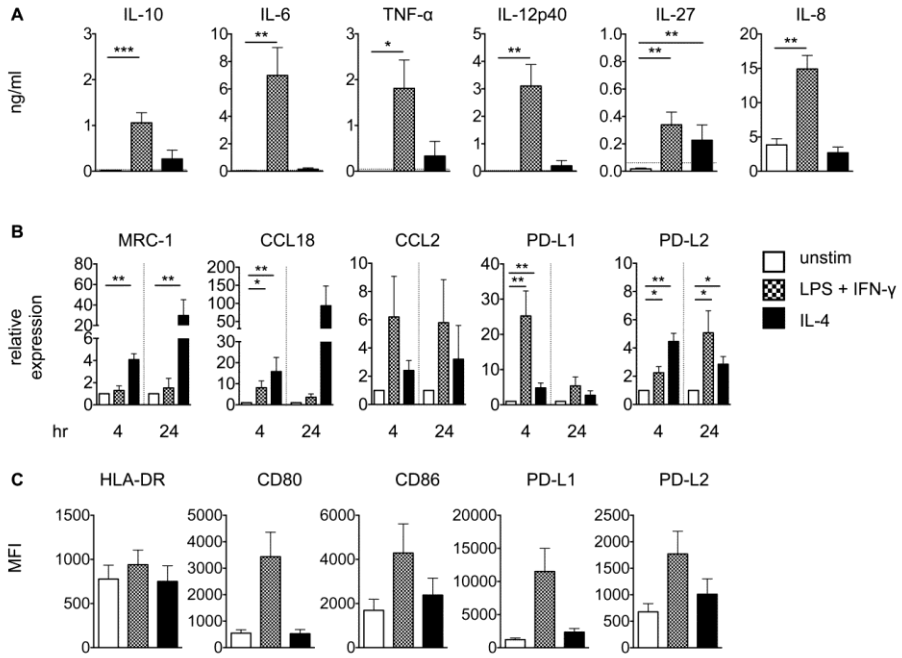


Figure 5-2. Macrophages respond appropriately to known polarising stimuli.
Macrophages from filaria non-endemic normal donors were left unstimulated or stimulated for 4 hs and 24 hs with LPS + IFN-γ or IL-4. A) Cytokine production was measured at 24 hs using ELISA. Results show pooled data from 4-6 experiments (n = 12-18). Horizontal dashed line indicates the limit of detection of the assay. B) mRNA expression was determined at 4 hs and 24 hs using RT-PCR. Results show pooled data from 2 experiments (n = 6). C) Surface expression of defined markers was measured at 24 hs by flow cytometry. Pooled data from 2 experiments (n = 6) are represented as the mean fluorescence intensity (MFI). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. * p<0.025, ** p<0.005, *** p<0.0005.

5.1.2. *B. malayi* female and Mf lysate act on monocytes and macrophages in a dose-dependent manner

A role for monocytes and macrophages in filariasis has been demonstrated in numerous mouse models and human studies[31,36,53,55,139,173,175,216-218]. To ascertain the importance of these cells in human filarial disease, it was first essential to establish whether monocytes and macrophages could be activated by filarial lysate, and to identify the optimal concentration of filarial lysate to use for future experiments. Thus monocytes and macrophages were stimulated for 24 hs with *B. malayi* female or microfilarial (Mf) lysate at varying doses (20, 10, 5 or 2.5 µg/ml), and protein production of IL-10 was measured by ELISA. IL-10 was used as a read-out because of its significance in filarial disease[70,78,170,219,220].

After 24 hs stimulation, monocytes produced high levels of IL-10 protein in response to *B. malayi* Mf lysate but not female lysate, in a clear, dose-dependent manner (**Figure 5-3**).

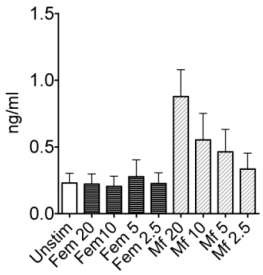


Figure 5-3. *B. malayi* Mf lysate but not female lysate acts on monocytes in a dose-dependent manner.
Monocytes from filaria non-endemic normal donors were stimulated for 24 hs with different doses (20-2.5 µg/ml) of *B. malayi* female lysate (Fem) or Mf lysate (Mf). IL-10 production was measured at 24 hs using ELISA. Results show data from 2 experiments (n = 6). Results represent mean ± SEM.

Stimulation of macrophages with varying doses of *B. malayi* female and Mf lysate produced a different result to that observed in monocytes. Macrophages produced IL-10 in a dose-dependent pattern after stimulation with *B. malayi* female lysate; however, this was relatively low compared with stimulation using Mf lysate (**Figure 5-4**). In contrast, *B. malayi* Mf lysate induced high levels of IL-10, which decreased as the dose was lowered.
In summary, a concentration of 20 µg/ml was chosen for future experiments employing *B. malayi* female and Mf lysate.

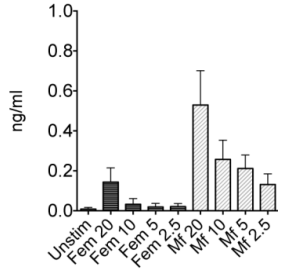


Figure 5-4. *B. malayi* female and Mf lysate act on macrophages in a dose-dependent manner.
Macrophages from filaria non-endemic normal donors were stimulated for 24 hs with different doses (20-2.5 µg/ml) of *B. malayi* female lysate (Fem) or Mf lysate (Mf). IL-10 production was measured at 24 hs using ELISA. Results show data from 2-3 experiments (n = 6-9). Results represent mean ± SEM.

5.1.3. Monocytes and macrophages stimulated *in vitro* with Mf lysate develop a distinct activation phenotype

To understand if *B. malayi* females or microfilariae act on monocytes and macrophages to induce immune modulation, monocytes isolated from buffy coats from filaria non-endemic normal donors were stimulated for 4 hs and 24 hs *in vitro* with 20 µg/ml *B. malayi* female or Mf lysate (**Figure 5-5**). In response to 24 hs stimulation with *B. malayi* female lysate, monocytes did not produce significantly different levels of any cytokines measured compared with unstimulated controls (**Figure 5-5A**). In response to 24 hs stimulation with *B. malayi* Mf lysate, monocytes produced significant and high levels of IL-10, IL-6, TNF-α and IL-8. Importantly, for both *B. malayi* female and Mf lysate, IL-12p40 was not induced, which is in clear contrast to the results observed earlier after stimulation with LPS + IFN-γ.
To assess the expression in monocytes of markers associated with an alternative or regulatory phenotype, mRNA expression of *MRC-1*, *CCL18*, *CCL2*, *PD-L1 and PD-L2* was analysed after 4 hs and 24 hs stimulation (**Figure 5-5B**). Expression of samples stimulated with *B. malayi* female or Mf lysate was normalised to unstimulated controls. *B. malayi* female lysate stimulation resulted in a slight, albeit significant, increase in *MRC-1* expression at 24

hs; the other markers measured were only slightly altered compared with unstimulated controls. *B. malayi* Mf lysate induced significantly higher levels of *CCL18* and *PD-L1* at 24 hs compared with unstimulated controls, while the other markers were not altered.
To determine whether the high mRNA level of *PD-L1* was reflected on a protein level, surface expression of PD-L1 was measured by flow cytometry. Expression of the markers HLA-DR, CD80, CD86 and PD-L2 was also measured (**Figure 5-5C**). In agreement with the PCR data, *B. malayi* female lysate did not alter expression of any markers measured. Importantly, PD-L1 was significantly upregulated in monocytes in response to *B. malayi* Mf lysate stimulation. Significant upregulation of PD-L2 was also observed after stimulation with *B. malayi* Mf lysate; however, HLA-DR, CD80 and CD86 were not significantly different from unstimulated controls.

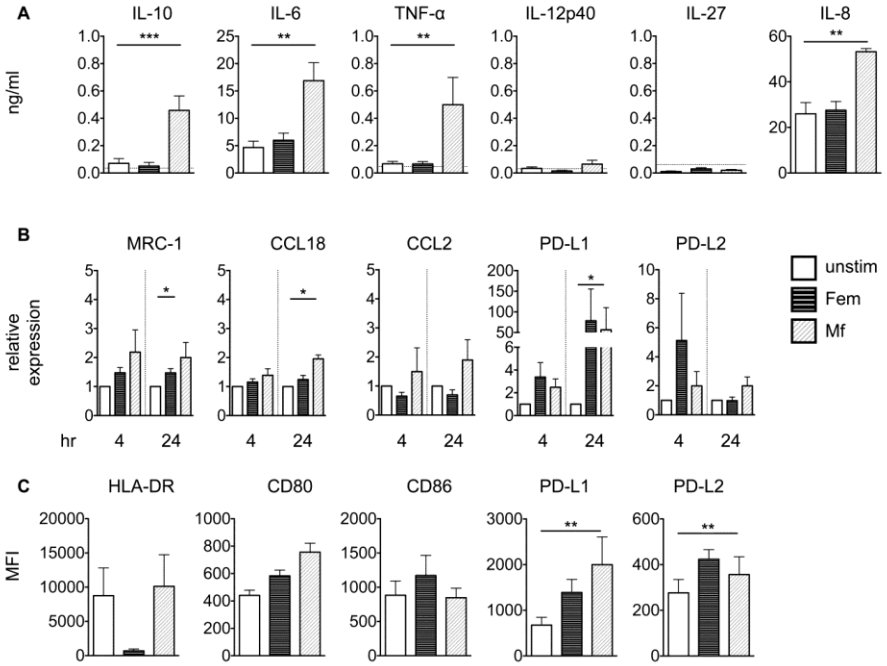


Figure 5-5. *B. malayi* Mf lysate acts on monocytes *in vitro* to induce a specific activation phenotype.
Monocytes were left unstimulated or stimulated for 4 hs and 24 hs with *B. malayi* female lysate (Fem) or Mf lysate (Mf). A) Cytokine production was measured at 24 hs using ELISA. Results show pooled data from 4-8 experiments (n = 12-24). Horizontal dashed line indicates the limit of detection of the assay. B) mRNA expression was determined at 4 hs and 24 hs using RT-PCR. Results show pooled data from 3 experiments (n = 8-9). C) Surface expression of defined markers was measured at 24 hs by flow cytometry. Pooled data from 2-4 experiments (n = 6-10) are represented as the mean fluorescence intensity (MFI). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. * p<0.025, ** p<0.005, *** p<0.0005.

In parallel, monocytes were differentiated to macrophages and stimulated with *B. malayi* female or Mf lysate (**Figure 5-6**)**.** In contrast to monocytes, *B. malayi* female lysate induced significantly upregulated levels of IL-10 and IL-8 compared with unstimulated controls (**Figure 5-6A**). IL-6, TNF-α, IL-12p40 and IL-27 were not induced by *B. malayi* female lysate stimulation of macrophages. Stimulation with *B. malayi* Mf lysate led macrophages to produce significantly higher levels of IL-10 and IL-8 compared with unstimulated controls, while IL-6, TNF-α, IL-12p40 and IL-27 were not induced.
On mRNA level, macrophages stimulated for 4 hs and 24 hs with *B. malayi* female lysate or Mf lysate did not alter expression of any of the markers analysed, compared with unstimulated controls (**Figure 5-6B**). This was also observed when surface expression of the markers HLA-DR, CD80, CD86, PD-L1 and PD-L2 was measured by flow cytometry at

24 hs; macrophages did not alter expression of these markers compared with unstimulated controls after stimulation with *B. malayi* female or Mf lysate (**Figure 5-6C**).

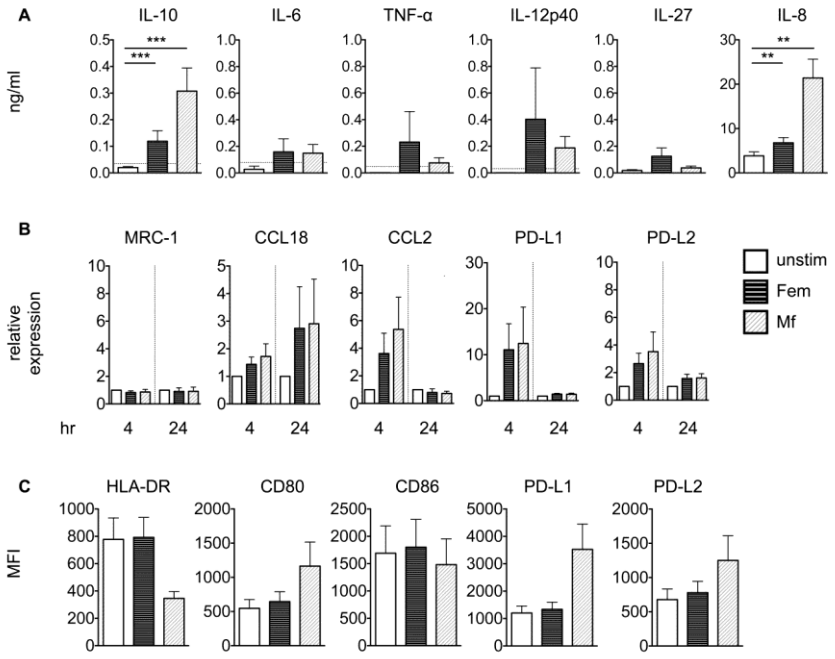


Figure 5-6. *B. malayi* female and Mf lysate act on macrophages *in vitro* to induce a specific activation phenotype.
Macrophages were left unstimulated or stimulated for 4 hs and 24 hs with *B. malayi* female lysate (Fem) or Mf lysate (Mf). A) Cytokine production was measured at 24 hs using ELISA. Results show pooled data from 3-7 experiments (n = 9-21). Horizontal dashed line indicates the limit of detection of the assay. B) mRNA expression was determined at 4 hs and 24 hs using RT-PCR. Results show pooled data from 2 experiments (n = 6). C) Surface expression of defined markers was measured at 24 hs by flow cytometry. Pooled data from 2 experiments (n = 6) are represented as the mean fluorescence intensity (MFI). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. ** p<0.005, *** p<0.0005.

It has previously been shown that microfilariae affect the survival of dendritic cells through apoptosis[67,221]. To determine whether *B. malayi* female or Mf lysate stimulation affected cell viability, monocytes or macrophages were stained with a dead cell exclusion dye (**Figure 5-7**). There was no difference in the percentage of viable cells in *B. malayi* female or Mf lysate-stimulated versus unstimulated monocytes or macrophages.

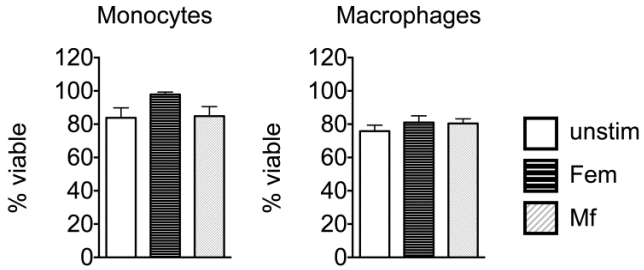


Figure 5-7. *B. malayi* female and Mf lysate do not affect cell viability.
Monocytes and macrophages were stimulated for 24 hs with *B. malayi* female (Fem) or Mf lysate (Mf) then stained with a dead cell exclusion dye and acquired by flow cytometry. Data are pooled from 2-4 experiments (n = 6-12). *p* values were calculated using the Wilcoxon signed-rank test.

Thus filarial lysate clearly acts on monocytes and macrophages from healthy donors who have no previous exposure to filarial parasites. The phenotype of monocytes did not alter drastically after stimulation with *B. malayi* female lysate. However, stimulation of monocytes with *B. malayi* Mf lysate induced expression of classically activated, alternatively activated and regulatory markers, with a more inflammatory and distinct profile compared with that observed after *B. malayi* female lysate stimulation. Most prominently PD-L1 and IL-10 were expressed at high levels in monocytes after stimulation with *B. malayi* Mf lysate.
Similar to monocytes, macrophages stimulated with *B. malayi* female lysate were not significantly different from unstimulated controls, although IL-10 and IL-8 were detected. While high levels of IL-10 were detected after stimulation with *B. malayi* Mf lysate, PD-L1 was not induced, a result that was in clear contrast to monocytes.

5.1.4. *B. malayi* female and Mf lysate-stimulated macrophages display impaired phagocytosis

To determine whether well-defined functions of monocytes and macrophages were modulated by stimulation with filarial lysate, monocytes and *in vitro* generated macrophages were stimulated for 24 hs with *B. malayi* female or Mf lysate. Subsequently, the phagocytic capacity was determined by measuring the phagocytosis of fluorescently labelled bioparticles, whereby unstimulated cells were used as a positive control, and set to 100% (**Figure 5-8**). Unstimulated cells plus cytochalasin D were used as a negative control. Monocyte phagocytic functions were not affected by *B. malayi* female or Mf lysate stimulation (data not shown). In contrast, *B. malayi* female and Mf lysate significantly inhibited phagocytosis in macrophages, reducing this function by approximately 30% for female lysate, and 20% for Mf lysate.

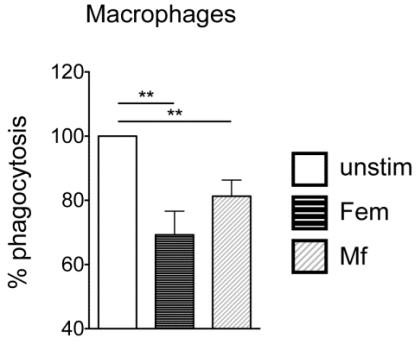


Figure 5-8. *B. malayi* female and Mf lysate inhibit macrophage phagocytic functions. *In vitro* generated macrophages were left unstimulated or stimulated for 24 hs with *B. malayi* female (Fem) or Mf lysate (Mf) after which the phagocytic capacity was determined by measuring the phagocytosis of fluorescently labelled bioparticles. Unstimulated cells were used as a positive control and were set to have a phagocytic capacity of 100%. Results for macrophages show pooled data from 3-4 experiments (8-11 donors). Data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. ** p<0.005.

5.1.5. *B. malayi* Mf lysate interferes with macrophage differentiation *in vitro*

As *B. malayi* Mf lysate, in contrast to female lysate, showed a continuous and distinct induction of IL-10 on monocytes and macrophages, it was decided to continue to work with Mf lysate to determine the effect of this stimulus on the differentiation process of monocytes to macrophages. In particular, it is feasible that microfilariae interfere with the differentiation of monocytes to macrophages *in vivo* during patent infection as a result of their shared anatomical locations in asymptomatically infected patients who carry microfilariae in the blood. Thus the phenotype and functions were determined of macrophages generated *in vitro* from $CD14^+$ monocytes in the presence of 20 µg/ml *B. malayi* Mf lysate (**Figure 5-9**). Mf lysate-differentiated macrophages did not alter expression of the maturation markers HLA-

DR, CD80 and CD86 or the macrophage markers CD11b and CD163 compared with macrophages generated in the absence of Mf lysate (**Figure 5-9A**). However, when macrophages generated in the presence of Mf lysate were washed and stimulated with 100 ng/ml LPS, there was a significant and selective inhibition of IL-6, TNF-α and IL-12p40 but not IL-10 expression, when compared with macrophages generated in the absence of Mf lysate (**Figure 5-9B**). Importantly this was not a result of decreased viability of Mf lysate-differentiated macrophages (**Figure 5-9C**).

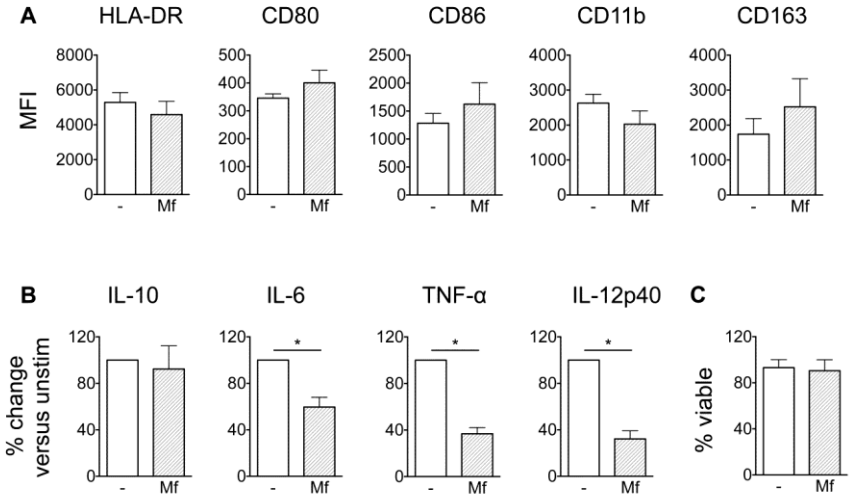


Figure 5-9. *B. malayi* Mf lysate interferes with the differentiation process of macrophages.
Monocytes were differentiated to macrophages with M-CSF in the absence or presence of 20 µg/ml Mf lysate. A) After 6 days, macrophages were analysed for surface expression of macrophage maturation markers by flow cytometry. B) After 6 days, the cell culture supernatant was removed and replaced with fresh medium plus 100 ng/ml LPS for 24 hs, to assess the capacity of Mf lysate-differentiated macrophages to produce cytokines, as measured by ELISA. C) A dead cell exclusion dye was used to stain cells that were subsequently acquired by flow cytometry. Pooled data from 2 experiments (n = 6). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. * $p<0.05$.

5.1.6. *B. malayi* Mf lysate-stimulated monocytes impair $CD4^+$ T cell proliferation and cytokine production

While *B. malayi* female lysate induced a stronger suppression of phagocytic functions of macrophages, *B. malayi* Mf lysate clearly targeted monocytes to induce a significant and high upregulation of the immunoregulatory markers IL-10 and PD-1, resulting in a distinct monocyte phenotype. This was of particular interest as monocytes colocalise with microfilariae in the blood of asymptomatically infected individuals[21]. Thus to determine whether the activation phenotype seen in monocytes stimulated with *B. malayi* Mf lysate may account for the hyporesponsiveness of $CD4^+$ T cells observed in *ex vivo* studies with PBMCs from AS patients[72,174,222-224] a coculture assay with autologous $CD4^+$ T cells was employed. To this end, autologous CFSE-labelled $CD4^+$ T cells were polyclonally stimulated and incubated with *B. malayi* Mf lysate-stimulated monocytes (**Figure 5-10**). T cell proliferation was significantly inhibited after coculture with *B. malayi* Mf lysate-stimulated monocytes (**Figure 5-10A**). Similarly, the production of IFN-γ, IL-13 and IL-10 was significantly inhibited in the supernatant of *B. malayi* Mf lysate-stimulated cultures compared with unstimulated control cultures (**Figure 5-10B**). Thus, Mf lysate-modulated monocytes showed a significantly impaired ability to stimulate $CD4^+$ T cell proliferation and effector functions.

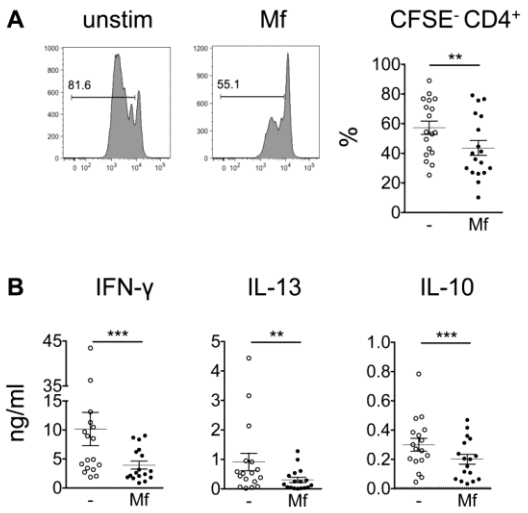


Figure 5-10. *B. malayi* Mf lysate-stimulated monocytes impair CD4$^+$ T cell proliferation and cytokine production.
0.5 x 10^6 CFSE-labelled CD4$^+$ T cells were incubated with 0.1 x 10^6 monocytes left unstimulated (open circles) or stimulated for 24 hs with *B. malayi* Mf lysate (closed circles) for 3 to 5 days. A) A representative flow cytometric analysis of CD4$^+$ T cells. Plots show dilution of CFSE over a 3-5 day period in T cells coincubated with unstimulated monocytes (left plot) or Mf lysate-stimulated monocytes (right plot). Graph shows the percentage of CD4$^+$ T cells that divided. B) Cytokine expression was measured in the culture supernatant by ELISA (pooled data from 4-6 experiments; n = 11-18). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. ** p<0.01, *** p<0.001.

In order to understand whether monocytes treated with microfilaria-derived ES products could alter T cell responses the coculture was repeated using monocytes stimulated with 20 µg/ml *B. malayi* microfilarial ES. Microfilarial ES-stimulated monocytes did not alter cytokine expression; there was a tendency to suppress proliferation of CD4$^+$ T cells although this did not reach statistical significance (**Figure 5-11**).

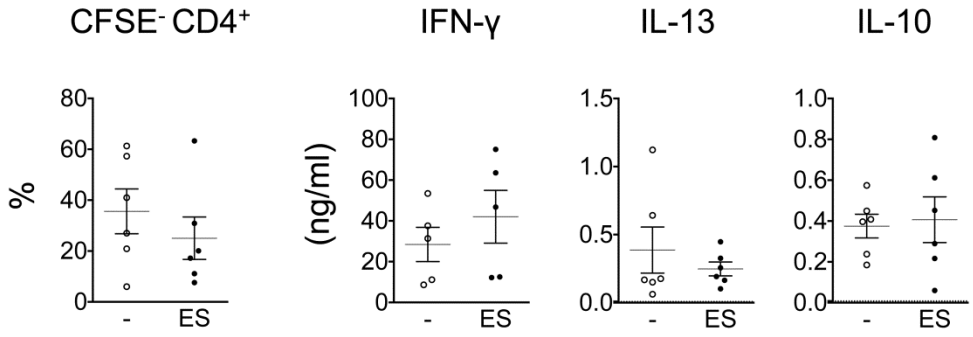


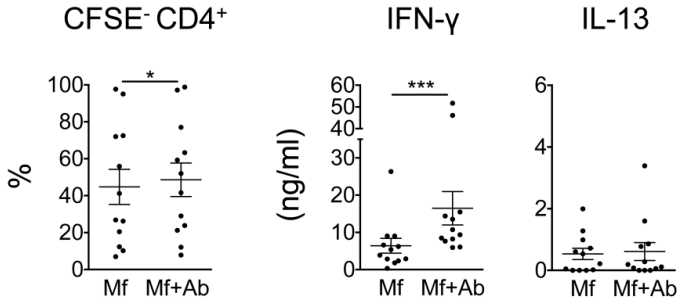
Figure 5-11. Monocytes stimulated with ES products from live *B. malayi* microfilariae do not show significant impairment of CD4$^+$ T cell proliferation or cytokine production.
0.5 x 10^6 CFSE-labelled CD4$^+$ T cells were incubated with 0.1 x 10^6 monocytes left unstimulated (open circles) or stimulated for 24 hs with *B. malayi* microfilarial excretory/secretory (ES) products (closed circles) for 5 days. A) Proliferation (measured as CFSE dilution) of CD4$^+$ T cells was measured by flow cytometry. B) Cytokine expression was measured in the culture supernatant by ELISA (pooled data from 2 experiments; n = 5-6). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test.

5.1.7. Neutralisation of IL-10 or PD-1 restores $CD4^+$ T cell IFN-γ production

As IL-10 and PD-L1 were significantly upregulated in *B. malayi* Mf lysate-stimulated monocytes, it was hypothesised that one of these molecules could be involved in inhibiting $CD4^+$ T cell functions as observed in **Figure 5-10**. Thus the coculture experiment was repeated with the inclusion of neutralising anti-IL-10 antibodies to block IL-10 signalling or anti-PD-1 antibodies to block PD-1:PD-L1 interactions (**Figure 5-12**).

While proliferation of T cells was elevated after neutralisation of IL-10 to a statistically significant level (p=0.016, **Figure 5-12A**), the biological difference in restoration was minimal (44.78% of T cells proliferated in response to Mf lysate-stimulated monocytes compared with 48.59% of T cells proliferating after IL-10 was neutralised). Proliferation of T cells was not changed after neutralisation of PD-1 (**Figure 5-12B**). IFN-γ production was restored in response to neutralisation of IL-10 (**Figure 5-12A**) or PD-1 (**Figure 5-12B**), while IL-13 responses were not restored in any case.

A. Neutralisation of IL-10



B. Neutralisation of PD-1

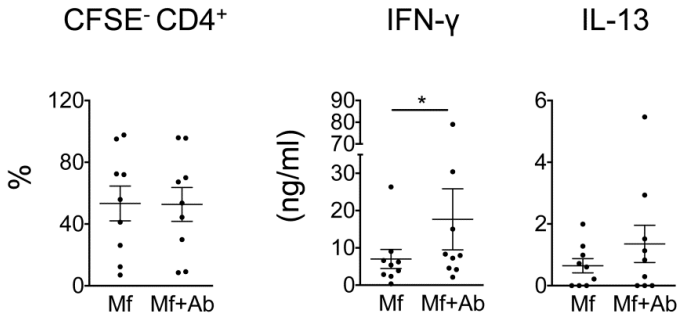


Figure 5-12. Neutralisation of IL-10 or PD-1 restores $CD4^+$ T cell IFN-γ production.
0.5 x 10^6 CFSE-labelled $CD4^+$ T cells were incubated for 5 days with 0.1 x 10^6 monocytes stimulated for 24 hs with *B. malayi* Mf lysate +/- neutralizing antibodies (Ab) for A) IL-10 (pooled data from 4 experiments; n = 12) or B) PD-1 (pooled data from 3 experiments; n = 9). Proliferation (measured as CFSE dilution) of $CD4^+$ T cells was measured by flow cytometry. Cytokine production was measured in the culture supernatant by ELISA. All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. * p<0.05, *** p<0.001.

To determine whether monocytes were actively expressing IL-10 upon the time of coculture, *B. malayi* Mf lysate-stimulated monocytes were assessed for *IL-10* mRNA expression by RT-PCR (**Figure 5-13**). After 24 hs stimulation with *B. malayi* Mf lysate, there was a trend for monocytes to express *IL-10* mRNA, although this did not reach statistical significance.

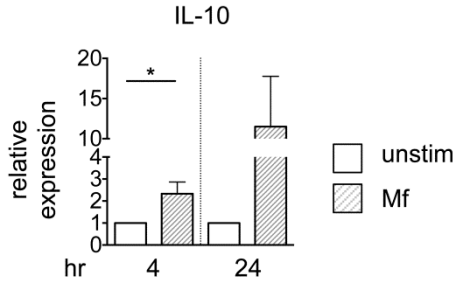


Figure 5-13. *B. malayi* Mf lysate-stimulated monocytes express *IL-10* mRNA at the time of coculture.
Monocytes were stimulated for 4 hs and 24 hs with *B. malayi* Mf lysate. mRNA expression was determined using RT-PCR. Results show pooled data from 3 experiments (n = 9). Data are represented as mean ± SEM. Stimulated condition was compared with paired unstimulated control. *p* values were calculated using the Wilcoxon signed-rank test. * $p<0.05$.

Together this data demonstrates that the observed regulatory phenotype of monocytes induced by *B. malayi* Mf lysate translates into an impaired stimulation of $CD4^+$ T cells and thus to stunted effector functions. This mechanism, at least for production of IFN-γ, is IL-10- and/or PD-1-dependent.

5.1.8. Stimulation of monocytes and macrophages with the filarial immunomodulator Bm-CPI-2 reflects the activation phenotype induced by *B. malayi* Mf lysate

Filarial cystatins are known to dampen antigen-dependent immune responses (reviewed elsewhere[98]). Numerous studies demonstrate the immunomodulatory potential of cystatin from *O. volvulus*, *A. viteae*, *L. sigmodontis*, *N. brasiliensis* and *B. malayi* on host cells[98,113,114,119,121,122,225]. In particular, Bm-CPI-2, a cystatin from *B. malayi*, has been shown to interfere with antigen processing[117]. Therefore the contribution of Bm-CPI-2 to the phenotype induced in monocytes and macrophages by *B. malayi* Mf lysate was determined.
To this end, monocytes from filaria non-endemic normal donors were stimulated for 4 hs or 24 hs with 20 µg/ml rBm-CPI-2 (**Figure 5-14**). In response to 24 hs stimulation with Bm-CPI-2, monocytes produced significant and high levels of IL-10, IL-6 and IL-8, compared with unstimulated controls (**Figure 5-14A**). TNF-α was also induced but the result did not reach statistical significance. IL-12p40 and IL-27 were not induced. To assess the expression in monocytes of markers associated with an alternative or regulatory phenotype, mRNA expression of *MRC-1*, *CCL18*, *CCL2*, *PD-L1* and *PD-L2* was analysed after 4 hs and 24 hs stimulation (**Figure 5-14B**). Bm-CPI-2 induced significantly higher levels of *CCL18* and *PD-L1* at 24 hs compared with unstimulated controls, and significantly reduced expression of *PD-L2* at 4 hs. The other markers were not or only slightly altered. This resulted in an overall phenotype that was similar to that produced in monocytes stimulated with *B. malayi* Mf lysate.
In parallel, monocytes were differentiated to macrophages and stimulated with Bm-CPI-2 (**Figure 5-15**)**.** In line with monocytes, Bm-CPI-2 induced significantly upregulated levels of IL-10, IL-6 and IL-8 in stimulated macrophages compared with unstimulated controls. In contrast to monocytes, macrophages also produced high and significant levels of TNF-α and IL-12p40 (**Figure 5-15A**). IL-27 was not induced by Bm-CPI-2 stimulation of macrophages. On mRNA level, macrophages stimulated with Bm-CPI-2 upregulated expression of *PD-L1* at 4 hs and 24 hs post stimulation compared with unstimulated controls. *PD-L2* was upregulated at 4 hs. *MRC-1*, *CCL18* and *CCL2* remained unchanged after stimulation with Bm-CPI-2 (**Figure 5-15B**).

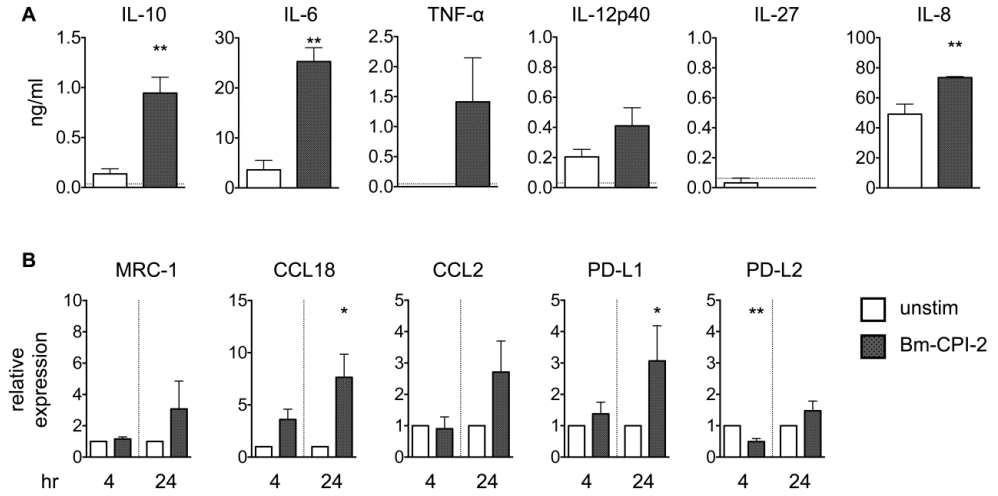


Figure 5-14. Stimulation of monocytes with rBm-CPI-2 reflects the activation phenotype induced by *B. malayi* Mf lysate.
Monocytes were left unstimulated or stimulated for 4 hs and 24 hs with rBm-CPI-2. A) Cytokine production was measured at 24 hs using ELISA. Horizontal dashed line indicates the limit of detection of the assay. B) mRNA expression was determined at 4 hs and 24 hs using RT-PCR. Results show pooled data from 3 experiments (n = 8). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. * p<0.05, ** p<0.01.

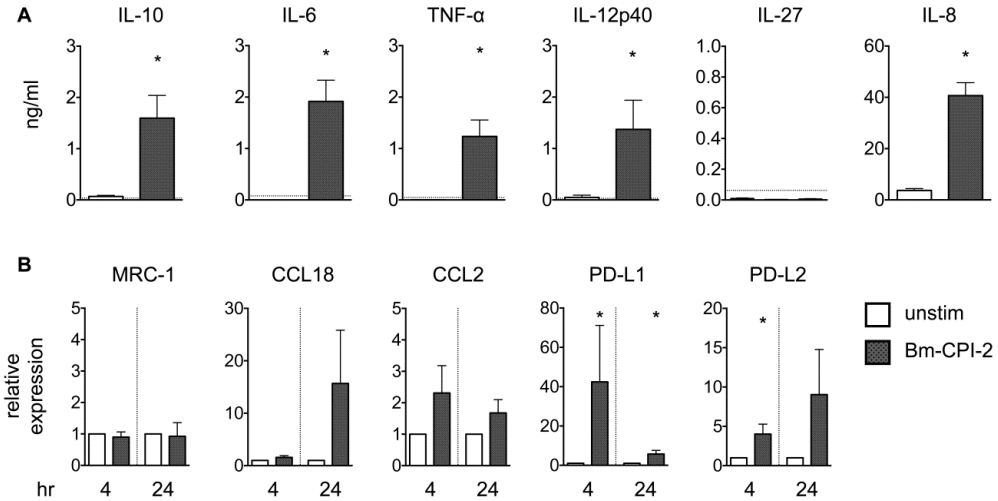


Figure 5-15. Stimulation of macrophages with rBm-CPI-2 shows an activation phenotype similar to *B. malayi* Mf lysate.
Macrophages were left unstimulated or stimulated for 4 hs and 24 hs with rBm-CPI-2. A) Cytokine production was measured at 24 hs using ELISA. Horizontal dashed line indicates the limit of detection of the assay. B) mRNA expression was determined at 4 hs and 24 hs using RT-PCR. Results show pooled data from 2 experiments (n = 6). All data are represented as mean ± SEM. *p* values were calculated using the Wilcoxon signed-rank test. * p<0.05.

5.2. Characterisation of immunological blood plasma parameters and the phenotype and function of monocytes from *W. bancrofti*-exposed donors

5.2.1. Filarial-specific antibody measurements confirm the diagnosis and classification of the *W. bancrofti*-exposed cohort

Samples collected from *W. bancrofti*-exposed donors were classified as being from an endemic normal donor (EN), a patient with chronic pathology (CP) or an asymptomatically infected patient (AS) after medical diagnosis by a qualified doctor together with tests for circulating filarial antigen (CFA, indicative of active infection) and night blood smears (see 4.2.2 for full details).

To further characterise the filaria-exposed cohort beyond clinical diagnosis and to align these characterisations with published literature, various immunological parameters typically used to class the different clinical groups were measured[20,24,177-179]. Thus total, *B. malayi* adult lysate (BmA)-specific and filarial cystatin-specific antibodies were measured in the plasma of donors who were classed as EN, CP or AS.

There was no difference in the amounts of total IgE, IgG1, IgG2, IgG3 or IgG4 between EN, CP or AS donors (**Figure 5-16A**). In contrast CP patients had significantly elevated levels of BmA-specific IgG2 compared with AS patients (**Figure 5-16B**). Furthermore there was a clear and significant increase in the levels of BmA-specific IgG4 in AS patients compared with EN donors (**Figure 5-16B**). To determine whether antibodies from *W. bancrofti*-exposed donors cross-reacted with cystatin from the filarial species *A. viteae*, the level of rAvCystatin-specific antibodies in the plasma were measured. Interestingly, the same pattern as seen for BmA-specific antibodies was observed when filarial cystatin-specific antibodies were measured, whereby AS patients displayed a significant increase in the level of IgG4 compared with EN donors (**Figure 5-16C**).

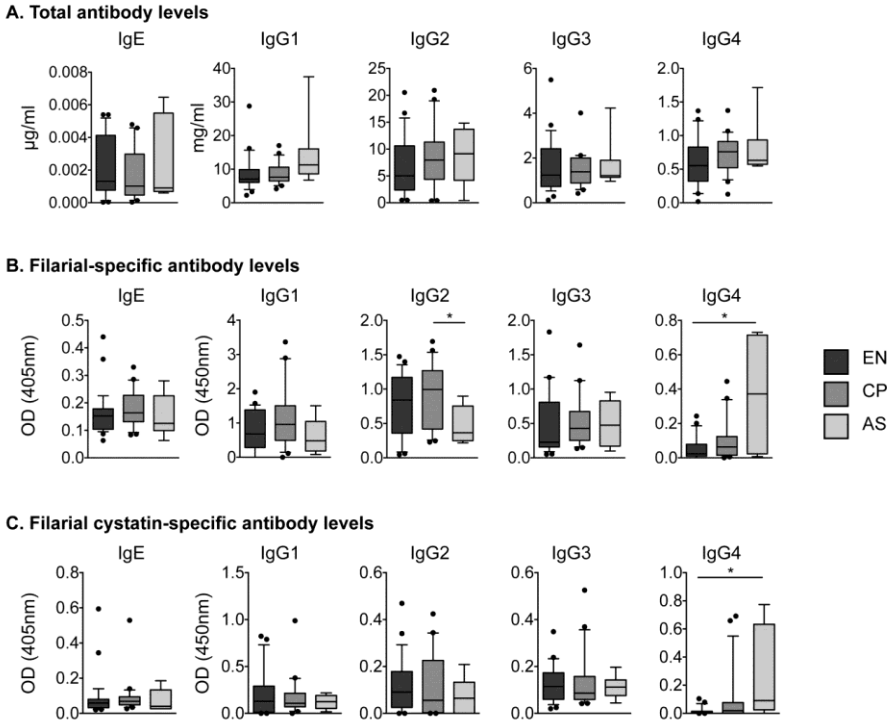


Figure 5-16. AS patients have significantly higher levels of *B. malayi* lysate-specific and filarial cystatin-specific IgG4.

A) Total antibodies, B) *B. malayi* adult lysate-specific antibodies, and C) filarial cystatin-specific antibodies were measured in the plasma by ELISA. EN, endemic normal (n = 28); CP, chronic pathology (n = 21); AS, asymptomatic infection (n = 7). *p* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons post-test. * $p<0.05$. Box-whisker plots demonstrate the 10th and 90th percentiles.

5.2.1. Monocytes from *W. bancrofti* AS patients have a regulatory phenotype

Stimulation *in vitro* with *B. malayi* Mf lysate induced a defined regulatory phenotype of monocytes that significantly upregulated the pro-inflammatory markers IL-6, TNF-α and IL-8 as well as the alternative/regulatory markers IL-10 and PD-L1. Therefore, it was hypothesised that in filaria-infected individuals, asymptomatically infected patients who exhibit circulating microfilariae in their blood develop a phenotype similar to that observed in *in vitro* experiments. Thus, RT-PCR was performed on monocytes isolated from PBMCs from EN, CP and AS donors (**Figure 5-17**). There was a trend for monocytes from AS patients to express elevated levels of the alternative/regulatory markers *PD-L1*, *PD-L2*, *IL-10*, *CCL18 and MRC-1* as well as the pro-inflammatory markers *IL-6*, *TNF-α* and *IL-12p40*. *IL-8* was significantly downregulated in AS patients compared with EN individuals while *CCL2* remained unchanged. *MRC-1* was significantly elevated in AS patients compared with CP donors. In contrast, there appeared to be little difference between CP and EN although *IL-10* and *IL-6* were significantly downregulated in CP compared with EN donors. Thus, monocytes isolated from PBMCs from AS patients recapitulate to a great extent the expression profile observed in *B. malayi* Mf lysate-stimulated monocytes from filaria non-endemic donors.

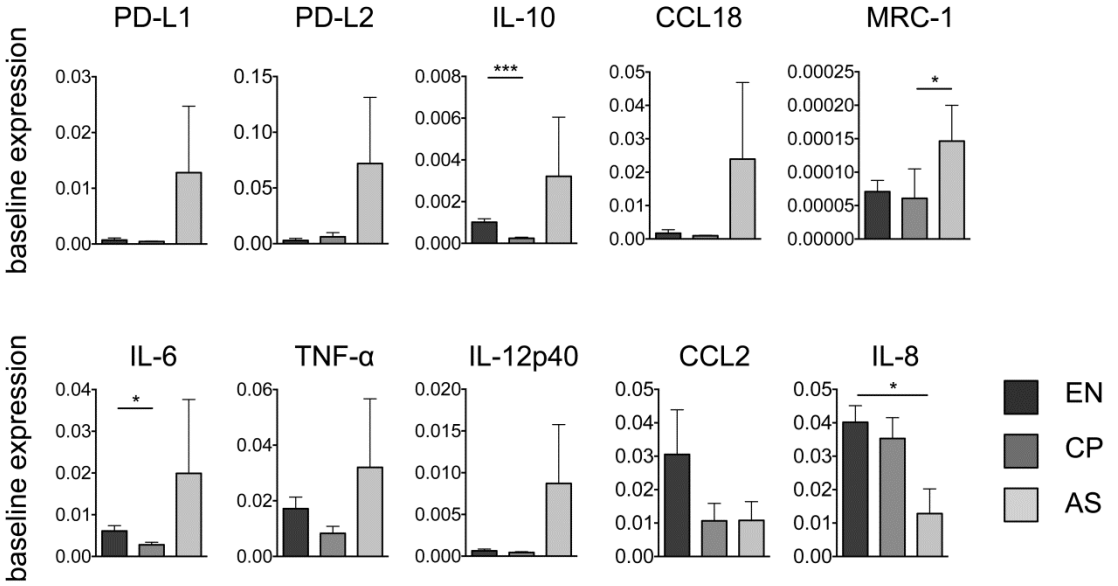


Figure 5-17. Monocytes from *W. bancrofti* AS patients have a regulatory phenotype at baseline.

Isolated monocytes from filaria-endemic donors were analysed *ex vivo* for mRNA expression. EN, endemic normal (n = 28); CP, chronic pathology (n = 21); AS, asymptomatic (n = 7). Data are represented as mean ± SEM. *p* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons post-test. * p<0.05, *** p<0.001.

5.2.2. Monocytes from filaria-exposed persons respond equally to *B. malayi*-specific stimuli regardless of the immunological background of the host

Monocytes from AS patients have been shown to be functionally defective in terms of TLR expression and function[36,226]. Thus the capacity of monocytes from AS patients to respond to *B. malayi* Mf lysate was established (**Figure 5-18**). After 24 hs without stimulation in culture, monocytes from EN, CP and AS donors responded equally in terms of protein production of IL-10, IL-6, TNF-α and IL-12p40. After 24 hs stimulation with Mf lysate, monocytes from all three groups responded by producing equal amounts of IL-10, IL-6, TNF-α and IL-12p40. Hence, there was no inherent defect in the ability of monocytes from EN, CP and AS donors to produce cytokines in response to Mf lysate.

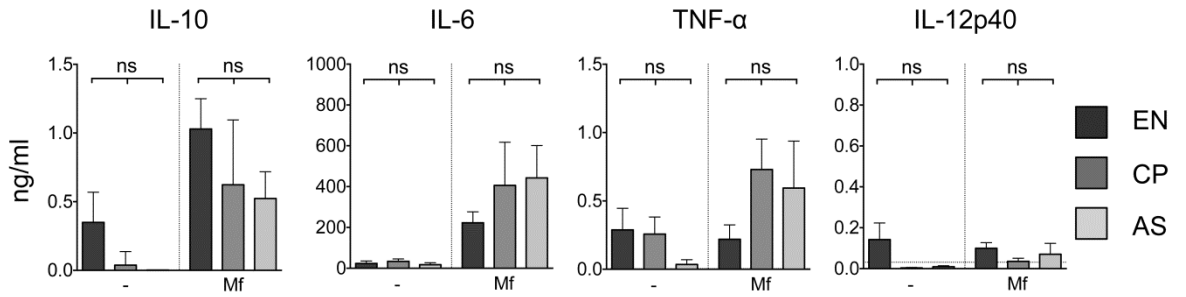


Figure 5-18. *B. malayi* Mf lysate acts on monocytes equally regardless of the immunological background of the host.
Monocytes from *W. bancrofti*-endemic donors were left unstimulated (-) or stimulated for 24 hs with *B. malayi* Mf lysate (Mf), after which cytokine production in the supernatant was measured by ELISA. EN, endemic normal (n = 14); CP, chronic pathology (n = 20); AS, asymptomatic infection (n = 4). Horizontal dashed line indicates the limit of detection. Data are represented as mean ± SEM. *p* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons post-test. ns, not significant.

5.3. Analysis of total IgG Fc *N*-linked glycosylation in *W. bancrofti*-exposed donors

5.3.1. *W. bancrofti* AS patients display a distinct antibody glycosylation profile

The cohort used for analysis of glycosylation profiles differed to that used in previous experiments (see **Table 4-2**). More plasma samples were collected and available than monocytes from *W. bancrofti*-exposed donors. Furthermore, in this case it was decided to categorise EN and CP donors based on having a matching age range to the AS group (the group with the lowest number of donors), as age has been demonstrated to affect antibody glycosylation[211,212]. Thus to ensure that the individuals were grouped correctly, the polyclonal and filarial-specific antibody levels of EN, CP and AS donors were determined (**Table 5-1**). There were no significant differences in polyclonal levels of IgE or IgG subclasses among the three groups. Similarly there was no difference in the levels of filarial-specific IgE, IgG1 or IgG3 among the three groups. However AS patients had significantly lower levels of IgG2 compared with CP patients, and significantly higher levels of IgG4 compared with EN donors (**Table 5-1**).

Table 5-1. Total and filarial-specific antibody levels in the plasma of the study cohort in Andhra Pradesh, South India, used to characterise the glycosylation profile.

	Endemic normal (EN)	Chronic pathology (CP)	Asymptomatic (AS)
Median polyclonal IgG (mg/ml) or IgE (µg/ml) levels (range)	IgE: 2.36 (0.17-5.40) IgG1: 7.30 (2.24-15.62) IgG2: 5.03 (0.49-20.54) IgG3: 1.16 (0.12-5.50) IgG4: 0.56 (0.02-1.37)	IgE: 1.81 (0.01-4.80) IgG1: 7.64 (3.08-19.33) IgG2: 6.43 (0.34-20.93) IgG3: 1.32 (0.45-4.02) IgG4: 0.76 (0.01-1.15)	IgE: 1.99 (0.60-6.46) IgG1: 9.96 (5.09-37.54) IgG2: 7.97 (0.38-14.84) IgG3: 1.18 (0.77-4.24) IgG4: 0.61 (0.39-1.72)
Median filarial-specific antibody levels in OD (range)	IgE: 0.15 (0.06-0.44) IgG1: 0.78 (0-2.45) IgG2: 0.73 (0.03-1.40) IgG3: 0.23 (0.05-1.83) IgG4: 0.02 (0-0.49)	IgE: 0.15 (0.08-0.33) IgG1: 0.96 (0-3.37) IgG2: 0.97 (0.19-1.84) IgG3: 0.38 (0.08-1.64) IgG4: 0.05 (0-0.45)	IgE: 0.14 (0.06-0.28) IgG1: 0.55 (0.08-1.50) IgG2: 0.33 (0.22-0.90) [a] IgG3: 0.36 (0.10-0.95) IgG4: 0.25 (0.01-0.73) [b]

Ig, immunoglobulin. OD, optical density, measured at 450 nm for IgG and 405 nm for IgE. EN, endemic normal (n = 25); CP, chronic pathology (n = 25); AS, asymptomatic infection (n = 8). *p* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons post-test. [a] $p<0.05$ AS versus CP; [b] $p<0.05$ AS versus EN.

To determine whether the glycosylation pattern of total IgG differed between EN, CP and AS donors, the *N*-linked glycosylation pattern of the Fc region of total IgG was examined using capillary electrophoresis laser-induced fluorescence (CE-LIF). This technique provides excellent and rapid separation and resolution of positional and linkage *N*-glycan isomers[227-231]. 25 EN individuals, 25 CP patients and 8 AS patients were analysed (**Figure 5-19**). **Figure 5-19A** shows a representative electropherogram from an endemic normal donor from South India, indicating all glycan structures present in the Fc region of total IgG. This profile clearly reflects the profile of a healthy individual described by others elsewhere[201,232,233]. Each *N*-glycan has a common pentasaccharide core that consists of two *N*-acetylglucosamine residues (GlcNAc) and three mannose residues that produce two antennae, denoted as (1-3) or (1-6). Variation is introduced on IgG *N*-glycans by addition of fucose (F) or bisecting GlcNAc (abbreviated as B) at the core, and GlcNAc, galactose (G) or *N*-acetyl neuraminic acid (sialic acid, S) to one or both of the terminal antennae[197].
The relative area was calculated for all peaks. For further analysis, only those structures were included that show the greatest expression differences in plasma IgG and therefore exert the greatest biological influence on the outcome of IgG responses. The structures included in the analysis were grouped as shown in **Figure 5-19B**. These structures have previously been identified as representing the corresponding individual peaks on the electropherogram[234]. The digalactosylated structure A2G2, representing less than 0.5% of

the IgG glycan pool[235], co-migrates with FA2(3)G1 and FA2B(6)G1 that are monogalactosylated, thus the three structures were integrated as a single peak and included in the calculation as a monogalactosylated glycan (G1). Similarly, FA2B(3)G1S1, FA2G2S1 and A2 co-migrate and were integrated as a single peak and classified as carrying one sialic acid (S1) because the contribution of A2 is minimal. Finally, FA2 and A2(6)G1 could not be distinguished; FA2 contributes to the majority of this value and thus the final value was classified as agalactosylated (G0).

As decreased galactosylation is typically correlated with the development of disease[236], the levels of galactosylation in total IgG were analysed in the three groups (EN, CP, AS). There was no difference among the three clinical groups in the levels of agalactosylated IgG (G0), or IgG that carried one (G1) or two (G2) galactose residues (**Figure 5-19C**). Increased sialylation (carrying either one or two sialic acid residues) is associated with anti-inflammatory responses[206], thus the levels of sialylation in total IgG were analysed in the three groups. There was no difference in the levels of monosialylated (S1) IgG (**Figure 5-19D**). However, AS patients had significantly lower levels of disialylated (S2) IgG compared with EN individuals and CP patients.

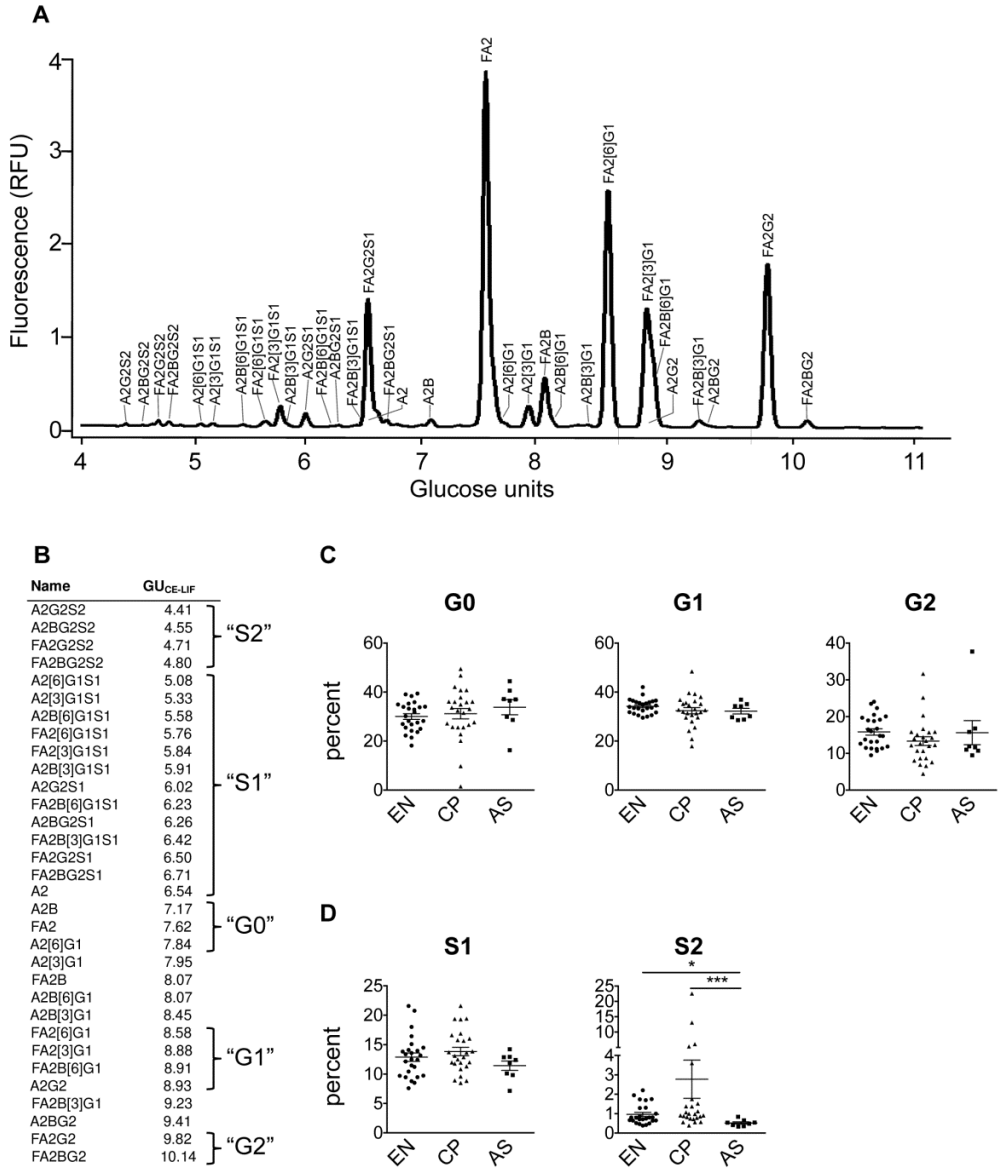


Figure 5-19. AS patients have significantly lower levels of disialylated IgG compared with EN donors or CP patients.

A) A representative CE-LIF electropherogram from an EN donor, indicating all structures present in the Fc region of IgG. RFU, relative fluorescence units. Structure abbreviations: *N*-glycans have a common pentasaccharide core denoted as A0 and consists of two *N*-acetylglucosamines (GlcNAc) and three mannose residues; F (1–6) linked core fucose; Ax: number of antennary GlcNAc attached to the trimannosyl core; B: bisecting GlcNAc; Gx: number of (1–4) linked galactose (G); [3]G1 and [6]G1 indicates that the galactose is either on the (α1–3) or (α1–6) antenna; Sx: number of *N*-acetyl neuraminic acid. B) Structural assignment of IgG *N*-glycans. CE-LIF migration times of *N*-glycans are given in glucose units (GU). Structures included in the final analysis are indicated. C) The percentage of IgG molecules that carry zero (G0), one (G1) or two (G2) galactose residues. D) The percentage of IgG molecules that carry one (S1) or two (S2) sialic acid residues. EN, endemic normal (n = 25); CP, chronic pathology (n = 25); AS, asymptomatic infection (n = 8). Data are represented as mean ± SEM. *p* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons post-test. * $p<0.05$, *** $p<0.01$.

6. DISCUSSION

6.1. *In vitro* stimulation of human monocytes and macrophages

6.1.1. Human monocytes and macrophages develop typical classically or alternatively activated characteristics under appropriate stimulation conditions

Monocytes and *in vitro* generated macrophages formed the basis of this project, thus it was essential to determine whether these cells respond to known polarising stimuli in a typical fashion in accordance with published literature. This would allow the comparison of monocytes or macrophages with a typical classically or alternatively activated phenotype with the cell populations stimulated with *B. malayi* filarial lysates. Filarial infections are known to stimulate a modified Th2 response, with a strong induction of macrophages that have a similar phenotype to macrophages induced by IL-4 stimulation[21,57,133]; in parallel microfilariae given alone (e.g. after injection in an animal model, in the absence of adult worms) have been shown to induce a more pro-inflammatory effect on the immune response than other lifecycle stages, such as the adults[237]. Thus, stimulating monocytes or macrophages with LPS + IFN-γ would provide a good standard with which to compare the induction of any classically activated genes that were expressed by *B. malayi* filarial lysate; similarly, stimulating monocytes or macrophages with IL-4 would provide a good standard with which to compare the induction of alternatively activated genes.

The results showed that stimulation of monocytes and macrophages with LPS + IFN-γ consistently induced expression of pro-inflammatory markers (IL-6, TNF-α, IL-12p40 and IL-8). LPS + IFN-γ is a hallmark stimulus that provides two signals to the cell, one through TLR4, CD14 and MD2 (by LPS) and one through the IFN-γ receptor[238]. This in turn leads to intracellular signalling and activation of NF-κB, AP-1, IRF3 and STAT1, which causes transcription of pro-inflammatory cytokines[238]. In line with classical activation is the observed increased expression of HLA-DR in monocytes stimulated with LPS + IFN-γ, required for efficient antigen presentation to activate the adaptive immune response.

In response to stimulation with LPS + IFN-γ, induction of the M2 pathway is generally not induced or is even downregulated, as demonstrated by the downregulation of MRC-1 in monocytes, and unchanged expression of this and other markers in macrophages. Thus the induction of CCL18 after stimulation with LPS + IFN-γ was surprising as this is described as an M2 marker for humans[153] and is clearly induced in monocytes and macrophages after stimulation with IL-4[239]. Nevertheless, secretion of CCL18 after stimulation of monocytes with LPS has been described[240], indicating that this marker may not be reliable for indication of M2 activation. Additionally, it has been described that IL-10 can induce expression of CCL18 in human macrophage cell lines; the large quantities of IL-10 observed in the current results may therefore have contributed to induction of this chemokine ligand[241].

Of the regulatory markers analysed, both monocytes and macrophages showed high levels of IL-10, PD-L1 and PD-L2 after stimulation with LPS + IFN-γ, although the expression of PD-L1 and PD-L2 did not reach a statistically significant level in macrophages. These results are reflected in the literature as LPS + IFN-γ stimulation of monocytes has previously been described to induce PD-L1 and PD-L2[239,242]. Similarly, it is well described that LPS stimulation of macrophages results in IL-10 production as an autocrine feedback mechanism essential to curb inflammatory responses[243].

Stimulation of monocytes and macrophages with IL-4 resulted in undetectable or suppressed (compared with unstimulated controls) levels of the pro-inflammatory cytokines measured, while the typical IL-4-induced markers, such as MRC-1 and CCL18, were readily detected[239]. This conforms with what is already known about IL-4 stimulation of monocytes and macrophages[133,148,244]. Furthermore, as has previously been described, IL-4 induced expression of PD-L1 and PD-L2[239,242]. CD86 was also upregulated on monocytes stimulated with IL-4, a result that has been reported elsewhere[245].

A major difference that was observed between monocytes and macrophages was the amount of cytokine produced by each population. Monocytes appeared to produce much greater quantities of IL-6 and IL-8 than macrophages; in contrast, IL-27 was not induced in monocytes but was present at high amounts in macrophages. This suggests that their individual phenotypes are differentially regulated. The lack of IL-27 production in LPS + IFN-γ-stimulated monocytes is intruiging as this cytokine has previously been reported to be induced in monocytes after LPS stimulation[246]. However, the authors of this paper used a cell line, THP-1, that may react differently to freshly isolated blood monocytes. Furthermore, they reported production of approximately 150 pg/ml of IL-27 in response to stimulation with 100 ng/ml LPS for 24 hs; this concentration is just above the limit of detection of the assay used in the current project, suggesting that use of a more sensitive assay could provide different results.
Examination of CCL2 (also known as macrophage chemotactic factor-1) indicated that this marker was not greatly altered by stimulation with IL-4 or LPS + IFN-γ. This marker was selected for its involvement in macrophage recruitment in murine filariasis[171]; however, the results suggest that CCL2 is regulated by stimuli other than IL-4 or LPS + IFN-γ.
To conclude, the results observed in monocytes and macrophages stimulated with IL-4 or LPS + IFN-γ confirmed that these cells respond as expected, in line with published literature.

6.1.2. *B. malayi* female and Mf lysate induce diverse responses in monocytes and macrophages, characterised by differential expression of M1, M2 and regulatory markers

The *B. malayi* female lysate-stimulated monocyte and macrophage populations varied only slightly with respect to their expression of M1, M2 and regulatory markers. Monocytes stimulated with female lysate did not upregulate any of the markers analysed compared with unstimulated controls. In contrast macrophages showed significant upregulation of IL-10 and IL-8. The previous results already demonstrated that these two cell populations respond differently with regard to IL-4 or LPS + IFN-γ stimulation. Thus, the fact that there are differences between monocytes and macrophages after stimulation with *B. malayi* female lysate was not surprising. Importantly, this highlights the fact that these two cell populations will presumeably respond differently to diverse stimuli *in vivo*, confirming that monocytes do not simply differentiate into macrophages with the same phenotype and functions. *B. malayi* Mf lysate-stimulated monocytes developed expression of numerous pro-inflammatory markers, including IL-6, TNF-α and IL-8, as well as expression of IL-10 and PD-L1. *B. malayi* Mf lysate-stimulated macrophages, in contrast to monocytes, only upregulated expression of IL-10 and IL-8. Therefore, the population that showed a clear response with a distinct phenotype was that of monocytes stimulated with *B. malayi* Mf lysate.
The expression of IL-10 in monocytes and macrophages is particularly interesting. A potential source of stimulation could come from an innate TLR signal such as from *Wolbachia*, the obligate endosymbiont found in all lifecycle stages of the filarial parasites *W. bancrofti* and *B. malayi* (as well as other filarial species)[247]. *Wolbachia* are known to induce inflammatory responses in monocytes by signalling through TLR2/6[248-250]. The levels of *Wolbachia* in Mf and female lysate used in this project were not known. *Wolbachia* have previously been described to give a positive result in a *Limulus* amoebocyte lysate (LAL) assay that measures endotoxin levels, and to contain LPS-like molecules[247,251]. *Wolbachia*, as gram negative bacteria, will presumeably react positively in a LAL assay. Nevertheless, the specificity of the assay and its ability to measure *Wolbachia*-derived endotoxin may vary, as the assay is based on reactivity to *E. coli*-derived endotoxin. It was later shown that *Wolbachia* signal in a TLR4-independent manner, wherein the authors suggested that the previous reports had been contaminated by environmental endotoxin[249,252]. The contribution of *Wolbachia* as an early inducer of IL-10 in monocytes could be tested by reproducing the same experiments but using a *Wolbachia*-negative parasite such as *A. viteae*. Nevertheless, the contribution of *Wolbachia* is questionable as monocytes but not macrophages stimulated for 24 hs with *B. malayi* female lysate (which also contains *Wolbachia*) produced a cytokine profile identical to that of unstimulated control monocytes (negligible IL-10, Figure 5-5). This

again highlights the differences in responses of monocytes and macrophages to filarial lysate stimulation. Furthermore, it indicates a microfilaria-derived factor other than *Wolbachia* to be responsible for the effects seen here with Mf lysate-treated monocytes.
The production of IL-10 in Mf lysate-stimulated monocytes could also be a result of the high levels of TNF-α observed after 24 hs stimulation with *B. malayi* Mf lysate. Other studies have demonstrated that TNF-α can induce IL-10 expression in monocytes[253,254]. In these studies, stimulation with LPS was required for initial induction of TNF-α. In the current results, *B. malayi* Mf lysate may provide this initial inflammatory stimulus; however, this is most likely not due to LPS as demonstrated by the negligible amount of LPS in Mf lysate preparations and absent induction of IL-12p40 in *B. malayi* Mf lysate-stimulated culture supernatants. In fact the lack of IL-12p40 induced by either Mf or female lysate clearly argues against a role for LPS in inducing the described phenotype.
The results for Mf lysate-stimulated monocytes indicate a phenotype that consists of a spectrum of pro-inflammatory, alternatively activated and regulatory markers, which is in line with numerous reports stating the mixed Th1/Th2 response that is often seen after exposure to microfilariae[67,174,237,255,256]. The phenotype (high IL-10, IL-6, TNF-α and low IL-12p40) also has some similarities with Type-II activated/M2b macrophages described for mice that develop in response to a combination of immune complexes plus TLR stimulation[148]; however, expression of MHC II and CD86, which are typically highly upregulated in M2b murine macrophages, remained unaffected in these results[257]. Monocytes isolated from filarial lysate-treated PBMCs from humans with asymptomatic filarial infection are characterised by increased expression of arg-1 and IL-10 compared with endemic normal controls and decreased levels of nitric oxide synthase (NOS, typically used as a classical activation marker in murine studies) compared with endemic normal controls[173]. Interestingly, in the current studies inducible NOS (iNOS) could not be detected in *B. malayi* Mf lysate-treated monocytes or macrophages *in vitro* or in monocytes from endemic patients *ex vivo* (data not shown). iNOS is historically difficult to detect, thus the differences observed here may lie in the techniques used[144]. Furthermore, murine markers cannot simply be translated into the human system[258,259]. As an example, reports indicate that arg-1 may not be a reliable marker for M2 monocytes or macrophages in humans as it is found in other cell types[260]. Human monocytes do not express arg-1 after stimulation with IL-4 and IL-13 (the prototypical inducers of alternative activation), unlike mouse macrophages[258]. Thus a number of markers were carefully selected for the current study, which have previously been described as typical markers for classical activation, alternative activation, or regulation in human monocytes and macrophages.

6.1.3. *B. malayi* female and Mf lysate inhibit phagocytosis

The data show that *B. malayi* female and Mf lysate inhibit phagocytic activity. As *B. malayi* female lysate was previously shown to have little effect on the phenotype of monocytes and macrophages, this was a surprising result. It has previously been reported that jirds injected with *B. malayi* L3 larvae develop adult worms and display peritoneal macrophages with decreased phagocytosis[261]. Nevertheless, the exact mechanism by which filaria decrease macrophage phagocytosis remains unclear.
It has previously been shown that *B. malayi* live microfilariae or microfilariae in a transwell do not induce phagocytosis in monocytes compared with monocytes exposed to M-CSF for 48 hours[239]. The authors of this study argued that microfilariae failed to promote phagocytosis. In contrast, it could be suggested that *B. malayi* microfilariae (or, in this case, *B. malayi* Mf lysate) directly inhibit phagocytosis of macrophages as a form of immunomodulation to promote parasite survival. In the current study, monocytes stimulated with *B. malayi* Mf lysate did not phagocytose differently compared with unstimulated monocytes. The location of macrophages in the tissues compared with monocytes in the blood may place macrophages in a more advantageous position to phagocytose. In another study, *W. bancrofti* endemic normal monocytes were incubated with serum from the different clinical groups (EN, CP, AS)[36]. Only monocytes incubated with serum from microfilaremic (AS) patients had reduced levels of spreading but not phagocytosis. Thus, future studies in

this area should determine in detail whether inhibition of phagocytosis actually translates into an increase in microfilarial survival, and which other cells or serum components contribute to microfilarial killing or phagocytosis *in vivo*.

6.1.4. *B. malayi* Mf lysate interferes with macrophage differentiation *in vitro*

In response to stimulation with *B. malayi* Mf lysate, macrophages upregulated high levels of IL-10 and had decreased ability to phagocytose bacteria. However, when *B. malayi* Mf lysate was added during the differentiation process of macrophages there was a significant drastic and selective impairment in the ability of macrophages to produce cytokines in response to LPS stimulation. Similar results have been published by Semnani *et al.* for monocyte-derived DCs stimulated with microfilarial antigen during the differentiation process[62]. Interestingly, the results presented in the current project were not caused by a reduction in cell survival or a change in cell activation markers. This is in contrast to results reported for DCs exposed to microfilariae that had significantly raised levels of apoptosis compared with unexposed DCs[221]. Importantly, the results in this project demonstrated that subsequent stimulation with LPS of macrophages differentiated in the presence of Mf lysate resulted in diminished IL-6, TNF-α and IL-12p40 production but not IL-10. This could be a result of the different signalling pathways activated within Mf lysate-differentiated macrophages and should be a subject of further studies. The selective impairment of expression of pro-inflammatory cytokines hints toward a possible involvement of NF-κB1 p50 homodimers, as shown previously[262].

6.1.5. *B. malayi* Mf lysate-modulated monocytes curtail $CD4^+$ T cell effector functions through IL-10- and PD-1-dependent mechanisms

Monocytes and macrophages that develop in helminth infections are believed to contribute to wound healing, regulation of Th1 and Th2 inflammation and expulsion of the parasite from the host (reviewed elsewhere[263]). Asymptomatically infected patients are the only filaria-exposed group in which monocytes in the blood come into contact with live microfilariae; thus, monocytes may be influenced at this early time point in their differentiation to contribute to immune regulation and thereby, the development of asymptomatic infection. Indeed it has been shown that *B. malayi* microfilariae target monocytes from filaria non-endemic normal donors to reduce transendothelial migration[264]. To this end, it was established that monocytes and macrophages from non-endemic normal donors stimulated with *B. malayi* Mf lysate *in vitro* develop a specific phenotype upon activation, and that these cells may influence either the adaptive or innate immune response, respectively.

B. malayi Mf lysate-stimulated monocytes could suppress $CD4^+$ T cell proliferation as well as IFN-γ and IL-13 cytokine production in an autologous coculture assay. T cells that received a polyclonal stimulus in the presence of *B. malayi* Mf lysate-treated monocytes displayed significantly reduced proliferation compared with control experiments. Furthermore, their ability to produce effector cytokines was significantly inhibited. This is in line with a previous report demonstrating that PBMCs from microfilaremic patients produce fewer Th1 and Th2 cytokines in response to live microfilariae compared with PBMCs from endemic normals[222].

PD-L1 and IL-10 were significantly induced by Mf lysate stimulation of monocytes and thus represented prime candidates responsible for the suppression of T cell responses. Interestingly, neutralisation of IL-10 or PD-1 led to a recovery of $CD4^+$ T cell IFN-γ but not IL-13 production. IL-10 has previously been described to be upregulated in adherent cells from patients with lymphatic filariasis[265] and in monocytes from patients harbouring tissue-dwelling filaria[248]. IL-10 has a well-defined role in filarial infections as an immunoregulatory cytokine that regulates both Th1- and Th2-derived inflammatory, potentially harmful responses[266]. PBMCs from asymptomatically infected patients spontaneously secrete significantly higher levels of IL-10 than PBMCs from patients with chronic pathology[255]. Induction of IL-10 is also associated with high levels of immune regulatory IgG4 in asymptomatic infection (reviewed elsewhere[78]). Thus the high levels of IL-10 observed in the current study may contribute to T cell suppression in asymptomatic infection.

PD-L1 has been described on monocytes stimulated with live microfilariae *in vitro*[239]. PD-L1 alongside its receptor PD-1 has an important role as a negative costimulator in numerous infection settings[267]. The high mRNA and surface expression of PD-L1 on *B. malayi* Mf lysate-stimulated monocytes support the idea that microfilaria-modulated monocytes may contribute to asymptomatic infection through this mechanism. Indeed IFN-γ responses were also restored after neutralization of PD-1, supporting a role for this molecule.
Neutralisation of IL-10 or PD-1 had only a minimal effect on the recovery of proliferation and had no effect on the restoration of IL-13, implying that other inhibitory mechanisms may be involved. Van der Werf *et al.* have previously shown that the PD-1:PD-L2 pathway is responsible for Th2 cell hyporesponsiveness in murine *L. sigmodontis* infection[268]. Nevertheless this pathway would have been similarly neutralised in the current assays through application of the anti-PD-1 antibody, suggesting that other mechanisms play a role in human T cell impairment as observed here. Other candidates that have been described in murine literature to suppress T cell responses in helminth or other Th2-related diseases through a monocyte/macrophage interaction include arginase[269,270] and RELM-α[271,272]; however, as mentioned previously, these molecules may not represent reliable options in the human system. Further studies describe the roles of retinoic acid or TGF-β in directing the development of Treg cells[273-275]; whether the $CD4^+$ T cells in this system have a regulatory phenotype should be investigated.

6.1.6. Stimulation of monocytes and macrophages with *B. malayi* cystatin (Bm-CPI-2) reflects the activation phenotype induced by *B. malayi* Mf lysate

Stimulation of human monocytes and macrophages with Bm-CPI-2 demonstrated for the first time that these cells are activated by this filarial immunomodulator to induce both M1, M2 and regulatory markers, specifically characterised by expression of IL-6, TNF-α, IL-10 and PD-L1. Uniquely, expression of these markers reflected to a certain extent the phenotype observed in monocytes and macrophages stimulated with *B. malayi* Mf lysate. In contrast to Mf lysate, macrophages also upregulated IL-12p40 after stimulation with Bm-CPI-2. There is some controversy about Bm-CPI-2 expression in the *B. malayi* lifecycle, as Gregory *et al.* described continuous expression throughout all lifecycle stages[276]. On the other hand Bennuru *et al.* describe an increase in expression in the microfilarial stage[277]. Both studies examined the transcriptional levels of this cystatin, thus to clarify this, future experiments should analyse protein production of Bm-CPI-2 to obtain a clearer picture on the actual levels *in vivo*. In *A. viteae* the microfilarial stages show the highest expression of the homologue AvCystatin, compared with other lifecycle stages[278]. Furthermore it was shown that IL-10 is induced in murine macrophages by *A. viteae*-derived AvCystatin[114]. Thus it would be interesting to investigate the contribution of Bm-CPI-2 in *B. malayi* Mf lysate-induced IL-10 expression. Furthermore, AvCystatin was shown to mediate IL-10- and macrophage-dependent immunomodulation in a mouse model of airway hyperreactivity[210]. Finally, it could be demonstrated that AvCystatin treatment *in vitro* of PBMCs from grass pollen-allergic patients resulted in a shift toward a Th1 phenotype, with significantly decreased production of IL-13, and increased levels of IFN-γ[279]. Thus, future experiments should determine the role of Bm-CPI-2 as an immunomodulator in filarial infection.

6.2. Characterisation of the antibody response and the phenotype and function of monocytes from *W. bancrofti*-exposed donors

6.2.1. IgG4 dominates the filarial-specific and cystatin-specific antibody response of *W. bancrofti* AS patients

To initially characterise the cohort, the levels of polyclonal and filarial-specific antibodies were determined. In contrast to general consensus, distinctively high levels of polyclonal IgG4 in AS or IgE in patients with pathology were not observed. A previous study has demonstrated in lymphatic filariasis on a polyclonal level that there is high IgG4:IgE ratio in AS patients and low IgG4:IgE ratio in CP patients[178]; however, another study provides conflicting results, suggesting that microfilaremic individuals have higher polyclonal IgE but equal IgG4 compared with endemic normals[280]. In contrast, the elevated levels of filarial-specific IgG4 in AS patients and of IgG2 in CP patients in this study reflects published literature[178,179,280,281]. This suggested a correct classification of the three groups, EN, CP and AS in line with reported literature[24,177,179] and based on the CFA content and diagnostic criteria used. Filarial-specific IgE was unexpectedly low in CP patients in the current study. Varying levels of IgE have been reported in AS and CP patients, that depend on the exact grouping of individuals (e.g. grouping patients according to severity of pathology, including CP patients who are microfilaremic, or using microfilarial count in place of CFA)[179,282]. The low numbers of patient samples in the current experiments unfortunately prevented a more detailed grouping of donors.

Of particular interest was the result that *W. bancofti*-exposed individuals had antibodies that recognised filarial cystatin, and that this response mirrored that of *B. malayi* filarial lysate-specific antibodies. Thus asymptomatically infected donors had extremely high levels of IgG4 that recognised filarial cystatin. As yet there are no publications describing the isolation of cystatin from *W. bancrofti*; however, it is clear that this species produces cystatin, as indicated by recognition of the molecule by antibodies from *W. bancrofti*-exposed donors. IgG4 is known to be induced at high levels in filarial infection together with IL-10, and has been described to have numerous immunomodulatory roles in other diseases[78,179]. Thus the high levels of cystatin-specific IgG4 in asymptomatically infected donors may highlight a further attempt for the parasite to induce the asymptomatic phenotype, by secreting high levels of cystatin.

6.2.2. The phenotype of monocytes from *W. bancrofti* AS patients reflects that observed in monocytes stimulated *in vitro* with *B. malayi* Mf lysate

Establishing the phenotype and function of *B. malayi* Mf lysate-stimulated monocytes and macrophages from non-endemic normal donors highlights these cells as targets of microfilarial immune modulation. To elucidate the exact phenotype of monocytes during infection, it was necessary to analyse the cytokine and marker profile of monocytes from endemic individuals *ex vivo*, without prior stimulation in the presence of other immune cells as done in previous studies[173]. Therefore monocytes from individuals with *W. bancrofti* asymptomatic infection were examined that had presumably interacted with microfilariae in circulation in the 12 hours prior to blood donation and monocyte isolation. As expected, in the absence of any external stimulation, only monocytes from this group produced the specific phenotype that was previously observed *in vitro*. Nevertheless in response to specific stimulation, it was found that monocytes from all three groups responded equally. This observation revealed that monocytes from all filaria-exposed donors principally had the capacity to react without an inherent defect in one of the patient groups.

In conclusion this study has elucidated the monocyte phenotype in patients with active filarial infection and the regulatory capacity of this cell. By directly acting on monocytes in the blood, microfilariae may regulate the antigen-specific T cell response ensuing in lymph nodes or tissues once monocytes are recruited. The extent to which this promotes parasite survival and transmission is unclear and should be further investigated.

6.3. *W. bancrofti* AS patients display a distinct antibody glycosylation profile characterised by decreased levels of disialylated IgG

The results from this project uniquely demonstrate that the levels of total IgG galactosylation did not differ among the two clinical manifestations of lymphatic filariasis (chronic pathology and asymptomatic infection) compared with infection-free endemic normals in a *W. bancrofti* endemic region in South India. However, persons with asymptomatic infection had significantly decreased levels of disialylated IgG compared with endemic normals and patients with pathology.

These results are in clear contrast to what was expected as decreases in galactosylation are typically associated with various inflammatory diseases[197,200]. This suggests that the underlying biochemical mechanisms may be different in lymphatic filariasis compared with other chronic inflammatory diseases. There is evidence to suggest that G0 IgG antibodies bind to activating FcγRs[236] as well as the mannose receptor (possibly through exposed mannose residues on the core glycan)[283,284] to induce pro-inflammatory responses. The current results demonstrated no differences in the level of galactose residues suggesting that this mechanism is not employed in lymphatic filariasis.

Interestingly, while the levels of monosialylated structures were equal among the three groups, the levels of disialylated structures in total IgG from asymptomatic patients were significantly lower than in endemic normals or patients with pathology. As patients with asymptomatic infection typically have a regulatory and Th2-type immune response, the opposite result, increased sialylation, was expected. In lymphatic filariasis, asymptomatic patients have patent, active infection, resulting in chronic antigenic stimulation of the immune response that is counteracted by parasite-induced regulatory and modified Th2-type responses. This is highlighted by the fact that asymptomatically infected patients, in contrast to all other individuals in a filaria-endemic area, are positive for circulating filarial antigen[280]. It has been described using a nephrotoxic serum nephritis model that antigenic stimulation results in decreased sialylation to induce a protective inflammatory response[207]. Nevertheless, if this is the case in lymphatic filariasis, the downstream result may be overridden in the face of the strong regulatory responses in asymptomatic patients.

Disialylated structures are typically found in <1% of the IgG pool[285], suggesting that high levels of these structures may be required to overcome activating FcγR signals to induce inhibitory FcγR signalling[286]. Sialylated IgG is known to bind FcγRIIB and dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN, known as SIGN-related 1 in mice) to induce anti-inflammatory responses (reviewed elsewhere[196]). Decreased binding of IgG to DC-SIGN, through lower levels of sialylation in the Fc region, may allow this receptor to become available for filarial antigens to bind. Previous reports demonstrate that helminth glycans bind DC-SIGN to induce immunomodulation in dendritic cells[287,288]. Thus decreased sialylation of IgG by the parasite may reduce competition for this receptor.

The source of disialylated IgG provides an avenue of further research, to determine whether one subclass in particular is responsible for carrying this phenotype. The use of protein A beads to purify IgG allows elution of IgG1, IgG2 and IgG4 but not IgG3[215], ruling out this latter subclass as a candidate. When more specific methods are employed to purify individual subclasses, glycans from IgG4 often produce a very low signal due to the low abundance of the antibody[289,290], suggesting that the phenotype observed in this study is unlikely to be occurring in IgG4. Additionally, it has been shown that IgG1 makes up the majority of intravenous immunoglobulin[291], which is known to have anti-inflammatory functions highly dependent on sialic acid in the Fc region[196]. Thus, future work should focus on the glycan contribution of each IgG subclass, with a particular focus on IgG1. Finally, further experiments should focus on analysing the glycosylation profile of antigen-specific IgG, which can vary greatly in comparison with total IgG[290].

7. LIMITATIONS OF THE STUDY

There were a number of limitations to this project that should be considered when analysing the data. Firstly, while *in vitro*-generated experiments offer a variety of avenues with which to explore a particular question, they are only an indication of what occurs in the tissues of an infected host. In particular, by generating macrophages *in vitro* it is assumed that the responses observed reflect those produced by macrophages *in vivo*. Macrophages are a heterogeneous cell population that has differing roles in diverse tissues. Therefore the macrophages that encounter filaria *in vivo* may differ in phenotype and function to macrophages generated *in vitro*. The method to generate macrophages *in vitro* in this study uses M-CSF and as such follows an established protocol that is used widely in this field[292-294]. However, it has been previously suggested that stimulation of macrophages with M-CSF may push these cells into an M2 direction[295,296]. This is an important point to consider as the protocol may generate macrophages that are more likely to express M2 markers in response to stimulation with *B. malayi* filarial lysates, which might otherwise not occur *in vivo*. Additionally macrophages come into contact with numerous other cytokines *in vivo*, including GM-CSF (thought to induce an M1 phenotype[295,296]). Nevertheless obtaining tissue macrophages from humans obviously has its own difficulties, and thus puts certain constraints and assumptions on the project.

In general, the results that are obtained using *in vitro*-generated experiments may not directly reflect the immune response of a filarial-endemic host, that while challenged with a long-living, immune modulating helminth species such as *W. bancrofti* or *B. malayi* also encounters daily bacterial, viral and allergenic challenges in the environment. Additonally, by using filaria non-endemic donors as the basis of the *in vitro*-generated experiments, one ignores (a) the genetic background of the host, e.g. polymorphisms that are abundant in endemic areas, and (b) the fact that filaria-endemic hosts have presumeably already been exposed to the parasites in utero. To perform these experiments, one must assume that these factors do not play a major role in the outcome of the host immune response.

Finally, a major limitation to these results from *W. bancrofti*-exposed donors was the access to only very low numbers of AS donors used for analysis of antibody responses, of *ex vivo* monocyte phenotype and for stimulation of filaria-endemic monocytes *in vitro*. Such low numbers of AS patients are in part due to the extensive and successful mass drug treatment effort in South India during the last decade leading to a low-endemicity for *W. bancrofti* and rare cases of active infection with accompanying microfilaraemia[38,39]. Nevertheless this highlights the need to continue work in this field to ensure reproduceability of these results and to gain mechanistic insights into filarial immunomodulation.

8. OUTLOOK

8.1. Cystatin as a filarial immunomodulator that contributes to the development of asymptomatic infection in lymphatic filariasis

Numerous smaller avenues for future studies have been described above; the outlook focuses on the contribution of filarial cystatin in the development of asymptomatic infection. Filarial cystatin is known to target both murine macrophages and human PBMCs to induce production of IL-10 and alter the host immune response[114,210,279]. The results presented in this project indicate that human monocytes and macrophages are susceptible to immunomodulation by Bm-CPI-2, and that this phenotype bears some similarities to monocytes and macrophages stimulated *in vitro* with *B. malayi* Mf lysate. Currently there are no publications that show the presence of cystatin in *W. bancrofti*; however, the related filarial species *O. volvulus*, *L. sigmodontis*, *A. viteae*, *B. malayi* and *N. brasiliensis* all produce cystatin with potential for host immune modulation[50,276]. Thus it can be assumed that *W. bancrofti* also produces cystatin (however, this should be verified by sequence analysis). Nevertheless this suggests that filarial cystatin may play an immunomodulatory role in *W. bancrofti* asymptomatically infected patients. Future experiments should determine the contribution of *B. malayi* cystatin as an immunomodulatory molecule produced by microfilariae during infection and present in Mf lysate. Currently it is unclear which *B. malayi* lifecycle stage expresses the highest levels of cystatin[276,277]. Thus it will be essential to determine the exact protein levels of Bm-CPI-2 secreted by microfilariae. Furthermore it would be interesting to analyse whether Bm-CPI-2-modulated monocytes or macrophages have decreased innate responses and/or alter $CD4^+$ T cell effector functions as described here for Mf lysate. Finally to determine the contribution of *B. malayi* cystatin to the immunomodulatory effects of Mf lysate, it would be necessary to deplete cystatin from *B. malayi* Mf lysate. This can be achieved by raising antibodies specific for recombinant Bm-CPI-2 in an animal model, after which the antibodies should be purified and tested for recognition of naturally occurring Bm-CPI-2. It is possible that these antibodies will also recognise the other cystatins expressed by microfilariae (Bm-CPI-1 and Bm-CPI-3). The *B. malayi* cystatin-specific antibodies can then be applied to *B. malayi* Mf lysate to deplete any cystatin present in its natural form within the lysate. Thus one can develop cystatin-depleted Mf lysate, and determine the exact contribution of cystatin to the immunomodulatory effects of *B. malayi* Mf lysate.

8.2. Determination of the glycosylation profile of cystatin-specific IgG and its subclasses, and the role of IgG with decreased sialylation

To analyse in depth the role of IgG glycosylation in lymphatic filariasis, future experiments should focus on analysing the glycan profile of antigen-specific IgG, which can vary greatly in comparison with total IgG[290], as well as the subclasses of IgG. In particular it would be interesting to analyse the glycan profile of filarial cystatin-specific antibodies that can be purified from plasma using recombinant cystatin. This experiment that would be facilitated by the use of recombinant cystatin that lacks glycosylation sites (production in a bacterial expression system omits this post-translational modification that could otherwise contaminate antibody glycosylation analyses[297]). Finally, one could develop recombinant IgG antibodies that carry a specific glycan profile (a procedure that has been described elsewhere[205]). This would allow one to observe *in vitro* whether decreased sialylation (as seen in AS patients) plays a role in host immune responses or parasite survival. As antibodies are clearly involved in the immune response to filarial worms, IgG Fc glycosylation may represent an as yet undescribed pathway in immunomodulation by this parasite. The experiments proposed here may shed light on this pathway.

ZUSAMMENFASSUNG

TITEL: Die Rolle von Monozyten und Makrophagen in der Ausprägung der Empfänglichkeit für Infektionen mit lymphatischer Filariose

HINTERGRUND UND ZIEL DER ARBEIT: Helminthen induzieren starke regulatorische und T-helfer 2 Immunantworten, indem Wirtszellen gezielt moduliert werden. Die Immunantwort des Wirtes gegen Erreger der lymphatischen Filariose entscheidet darüber, ob ein Individuum resistent ist oder erfolgreich infiziert wird. Darüberhinaus führt die Infektion entweder zu einer klinisch asymptomatischen Manifestation, welche die Transmission des Parasiten begünstigt oder zu chronischer Pathologie, welche für die hohe Morbidität von lymphatischer Filariose-Patienten verantwortlich ist. Monozyten und Makrophagen tragen zur helminthen-induzierten Dysfunktionalität der Immunantwort bei, indem sie von Mikrofilarien in Blut und Gewebe moduliert werden. Während einer patenten Infektion treffen Monozyten im Blut auf zirkulierende Mikrofilarien ausschliesslich in asymptomatischen Patienten, welche sich durch eine Hyporeaktivität ihrer Immunantwort auszeichnen. Weiterhin induzieren Helminthen regulatorische Antikörperisotypen, welche einen Einfluss auf den Verlauf der Infektion haben. Aus anderen Krankheitsmodellen ist zudem bekannt, dass eine veränderte Glykosylierung der IgG Fc Region mit einer Pathologie einhergeht. Das Ziel dieser Arbeit war zu untersuchen, ob Mikrofilarien Monozyten aus dem Blut oder Makrophagen zu einem regulatorischen Phänotyp induzieren können, welche modulierte angeborene und adaptiven Immunantworten vermitteln. Weiterhin sollte das IgG Glykosylierungsprofil von *Wuchereria bancrofti* infizierten Individuen bestimmt werden, um einen eventuellen Einfluss der Glykosylierung auf das Krankheitsbild zu ermitteln.

ERGEBNISSE: Monozyten und *in vitro* generierte Makrophagen von non-endemischen Individuen zeigen einen drastisch veränderten Phänotyp nach Stimulation mit Mikrofilarien (Mf) Lysat; adultes Weibchenlysat vermittelte keine deutlichen Veränderungen. Mit Mf Lysat stimulierte Monozyten entwickelten einen definierten regulatorischen Phänotyp mit erhöhter Expression von IL-10 und PD-L1. Interessanterweise wurde dieser Phänotyp in Monozyten von *W. bancrofti* asymptomatisch infizierten Individuen, jedoch nicht in Patienten mit chronischer Pathologie oder uninfizierten endemischen Kontrollindividuen rekapituliert. Interessanterweise sind Mf Lysat-stimulierte Monozyten von nicht-endemischen Donoren nicht in der Lage $CD4^+$ T-Zellen adäquat zu stimulieren. Im Gegensatz zu Kontrollmonozyten konnte eine signifikante Inhibierung der Proliferation und Zytokinexpression der polyclonal stimulierten $CD4^+$ T-Zellen festgestellt werden. Dabei war die Inhibierung der IFN-γ Expression sowohl abhängig von IL-10 als auch PD-1, wie in Neutralisationsversuchen gezeigt werden konnte. Weiterhin zeigten Mf Lysat-stimulierte Makrophagen ebenfalls erhöhte IL-10 Synthese mit gleichzeiteiger reduzierter Phagozytose. Zudem führte die Generierung von Makrophagen in der Anwesenheit von Mf Lysat zu einer Makrophagenpopulation mit stark verminderter Fähigkeit auf einen LPS-Stimulus zu antworten. Schliesslich konnte die Analyse der N-Glykane der IgG Fc Region von *W. bancrofti* exponierten Individuen zeigen, dass es zwar keinen Unterschied in der Galaktosylierung der drei endemischen Gruppen gibt, asymptomatisch infizierte Individuen aber eine signifikant erhöhte Sialylierung der Fc Region in totalem IgG aufweisen.

SCHLUSSFOLGERUNG & BEDEUTUNG: Diese Arbeit konnte demonstrieren, dass Mf Lysat einen regulativen Phänotyp von Monozyten induziert, welche eine verminderte T-Zell stimulatorische Kapazität aufweisen. Dieser Phänotyp konnte ebenfalls in asymptomatischen Mikrofilarienträgern nachgewiesen werden. Diese Daten deuten darauf hin, dass die typischerweise beobachtete Immunsuppression in asymptomatisch Infizierten zum Teil auf durch Mikrofilarien modulierte Monozyten zurückgeht, welche sich in Abhängigkeit von IL-10 und PD-1 entwickelt. Zusammen mit der beobachteten Suppression natürlicher Immunantworten könnte dies der generellen Herunterregulierung von Immunantworten in asymptomatisch Infizierten zu Grunde liegen.

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PUBLICATIONS & SCIENTIFIC CONTRIBUTIONS

Publications

N.L. O'Regan & S. Steinfelder, G. Venugopal, G.B. Rao, R. Lucius, A. Srikantam, S. Hartmann. *Brugia malayi* microfilariae induce a regulatory monocyte/macrophage phenotype that suppresses innate and adaptive immune responses. *PLoS Neglected Tropical Diseases* **8**(10):e3206.
doi: 10.1371/journal.pntd.0003206

N.L. O'Regan, S. Steinfelder, C. Schwedler, G.B. Rao, A. Srikantam, V. Blanchard, S. Hartmann. Filariasis asymptomatically infected donors have lower levels of disialylated IgG compared with endemic normals. *Parasite Immunology* **36**: 713-720.
doi: 10.1111/pim.12137

E. Danilowicz-Luebert & **N.L. O'Regan**, S. Steinfelder, S. Hartmann (2011). Modulation of specific and allergy-related immune responses by helminths. *Journal of Biomedicine and Biotechnology*. 2011:821578.
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Contributions at scientific meetings

N.L. O'Regan, S. Steinfelder, R. Lucius, A. Srikantam, S. Hartmann. Monocytes as determinants of susceptibility in lymphatic filariasis. *GRK1673 retreat, 11th - 12th November 2013, Berlin, Germany. Poster presentation*.

N.L. O'Regan, S. Steinfelder, R. Lucius, A. Srikantam, S. Hartmann. Monocytes as determinants of susceptibility in lymphatic filariasis. *17th annual Woods Hole Immunoparasitology meeting, 28th April - 1st May 2013, Woods Hole, MA, USA. Oral presentation.*

N.L. O'Regan & N. Dittrich. Living and working in Hyderabad – a scientific contribution. *Young Investigators Meet, 15th February 2013, Frankfurt, Germany. Organised by the Embassy of India in Berlin. Oral presentation.*

N.L. O'Regan, S. Steinfelder, R. Lucius, A. Srikantam, S. Hartmann. Monocytes and macrophages in lymphatic filariasis: molecule determinants of susceptibility. *GRK1673 retreat, 1st - 2nd November 2012, Berlin, Germany. Oral presentation*.

N.L. O'Regan, S. Steinfelder, R. Lucius, A. Srikantam, S. Hartmann. Monocytes and macrophages: determinants of susceptibility in lymphatic filariasis. *Global challenges of chronic tropical infections ZIBI/GRK1673 symposium, 18th - 19th June 2012, Berlin, Germany. Poster presentation.*

N.L. O'Regan, S. Steinfelder, R. Lucius, A. Srikantam, S. Hartmann. Monocytes and macrophages: determinants of susceptibility in filariasis. *Annual meeting of the German Society for Parasitology, 14th - 17th March 2012, Heidelberg, Germany. Oral presentation*.

N.L. O'Regan, S. Steinfelder, R. Lucius, A. Srikantam, S. Hartmann. Monocytes and macrophages in lymphatic filariasis: molecule determinants of susceptibility. *GRK1673 retreat, October 2011, Berlin, Germany. Oral presentation*.

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*not really, I would probably leave him with Alice or Sanjay, or literally anyone else, where he would be more likely to survive.

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Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Noëlle Louise O'Regan

Berlin, den 29.08.14