Aus dem Institut für Immunologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Brugia malayi cystatin induced immunomodulation on human monocytes and macrophages and identification of immune gene polymorphisms associated with lymphatic filariasis

Inaugural-Dissertation
zur Erlangung des akademischen Grades
philosophiae doctor (Ph.D.) in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Gopinath Venugopal
Immunoparasitologe aus Chennai, Indien

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Zweiter Gutachter:	Prof. Dr. med. Ralf R. Schumann
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Dedicated to my parents

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AAM alternatively activated macrophage
APC antigen-presenting cell
Arg arginase
AS asymptomatically infected
AvCystatin cystatin from *Acanthocheilonema viteae* (= Av17)
Bm-CPI-2 cystatin from *Brugia malayi*
B. malayi (*Brugia malayi*)
B. pahangi (*Brugia pahangi*)
BSA bovine serum albumin
CAM classically activated macrophage
CCL chemokine C-C motif ligand
CD cluster of differentiation
CFA circulating filarial antigen
CFSE carboxyfluorescein succinimidyl ester
CP chronic pathology
CTLA cytotoxic T-lymphocyte antigen
DC dendritic cell
DEC diethylcarbamazine
ELISA enzyme-linked immunosorbent assay
EN endemic normal
E/S excretory/secretory
F female
FACS fluorescence-activated cell sorting
FMO fluorescence minus one
FoxP3 forkhead box protein P3
H. polygyrus (*Heligmosomoides polygyrus*)
hM-CSF human macrophage colony-stimulating factor
hrs hours
HRP horseradish peroxidase
IFN-γ interferon gamma
Ig immunoglobulin
IL interleukin
iNOS inducible nitric oxide synthase
iTreg inducible regulatory T cells
JAK Janus family tyrosine kinase
L1 first larval stage
L3 third larval stage
L4 fourth larval stage
LF lymphatic filariasis
L. sigmodontis (*Litomosides sigmondontis*)
LPS lipopolysaccharide
M male
MAPK mitogen-activated protein kinase
M-CSF macrophage colony-stimulating factor
MDA mass drug administration
Mf microfilariae
MF microfilariae lysate
MFI mean fluorescence intensity
MHC major histocompatibility complex
min minutes
MIF macrophage migration inhibitory factor
MRC mannose receptor C type

nTreg natural regulatory T cells
NO nitric oxide
ns not significant
O. volvulus (*Onchocerca volvulus*)
PBMC peripheral blood mononuclear cells
PBS phosphate-buffered saline
PD-1 programmed death protein-1
PD-L programmed death-ligand
r recombinant
RELM resistin- like molecule
SEA *Schistosoma mansoni* soluble egg antigen
sec seconds
SEM standard error of the mean
STAT signal transducers and activators of transcription
TGF transforming growth factor
TNF tumour necrosis factor
Th T helper
TLR Toll-like receptor
Treg T regulatory
VEGF vascular endothelial growth factor
WHO World Health Organization
W. bancrofti (*Wuchereria bancrofti*)
WoLP *Wolbachia* lipoproteins
WSP *Wolbachia* surface protein

1. Introduction

1.1 Helminth parasites

About one-third of the world population is infected with helminth parasites. Helminths are parasitic worms that live at the expense of their hosts and develop interspecific non-mutual symbiotic relationships with their partners. About 50 % of all species live parasitic or go through a parasitic life stage at least once in their lifetime. Historically, there were many evidences from the ancient writings of Hippocrates, Egyptian medical papyri, and the Bible suggesting the characteristic clinical features of helminth infections. Due to the widespread nature of helminths in livestock, they are not only responsible for causing major agricultural damage, but also considered to be a detrimental factor for many debilitating diseases and syndromes in human and animal hosts (Hotez et al., 2008).

Generally, helminths are multicellular eukaryotic invertebrates characterized by three taxonomic groups: **cestode tapeworms**, **trematode flukes,** and **nematode roundworms**. Cestodes (tapeworms) are long, flat, ribbon-like bodies with a single anterior holdfast organ (scolex) and contain three to several thousand segments called proglottids (length 2-3mm to 10mm). Trematodes (flukes) are small, flat, leaf-like bodies with anterior and ventral suckers and blind sac-like intestinal tracts; usually, the size of an adult fluke can range from 1 mm to more than 10 cm in length. On the other hand, nematodes (roundworms) are long, thin, unsegmented tube-like bodies with anterior mouths and longitudinal digestive tracts. Both adult and larval roundworms are bisexual, cylindrical worms and they inhabit intestinal and extra-intestinal sites. (Gelderblom, 1996; Hotez et al., 2008). Helminths are also classified on the basis of the morphology of eggs, larval, and adult stages: mainly the ***plathyhelminthes*** or flatworms (platy from the Greek root meaning “flat”) include flukes and tapeworms and the ***Nematoda*** or roundworms (nemato from the Greek root meaning “thread”). These groups are further subdivided based on their ability to dwell in certain organs of the host, e.g., lung flukes, extraintestinal tapeworms, and intestinal roundworms. Also, helminths have various invasion routes, which include the skin (schistosomes and hookworms), filarial worms via mosquito bite), and through the gastrointestinal tract (Gelderblom, 1996).

Typically, helminths have the capacity to modulate the host immune system to maintain their long-term survival within the host. Presumably, the ability of helminths to establish a long-term chronic infections within their hosts entails interactions with various immune cells and subsequent immunomodulation leading to a failed eradication mechanism towards the parasites (Allen and Maizels, 1996, 2011; Anthony et al., 2007; McSorley and Maizels, 2012).

1.2 Nematodes

Nematodes are considered to be one of the important clusters of metazoans and are mostly elongated, round, non-segmented and bilaterally symmetrical worms. Usually, the lengths of the nematodes ranges between a few millimetres and one meter. Although the total estimated number of nematodes is to be around 1 million species, only 25000 species of nematodes have been described so far. Generally, nematodes are found in all types of animals and plants and that might be due to its overall conquering ability to all kinds of habitats. Until now, about 138 human pathogenic species of nematodes have been described. Globally, about 2 billion people are infected with intestinal nematodes, while extra-intestinal nematodes infect approximately 200 million people (Johnston et al., 2014; Cooper and Eleftherianos, 2016). Table 1 summarizes briefly the global prevalence and distribution of major human nematode infections (Hotez et al., 2008).

1.3 Lymphatic filariasis (LF)

This thesis work focusses on molecular determinants for susceptibility to lymphatic filariasis. Lymphatic filariasis (LF) is a well-known neglected disease of the tropics and subtropics and is caused by infections with helminths of the nematode subfamily *Filarioidea,* comprised of the families *Filariidae* and *Onchocercidae,* including numerous important species which are known to cause major diseases in humans (Otsuji, 2017).

As per the World Health Organization (WHO), LF is listed as one among the seventeen Neglected Tropical Diseases (NTDs) (Johnston et al., 2014) and three species of filarial nematodes are responsible for causing LF in humans; ***Wuchereria bancrofti (W. bancrofti)***, ***Brugia (B. malayi) malayi*** and ***Brugia timori (B. timori)***. LF is still considered to be one of the leading causes of physical disability worldwide with approximately 120 million people currently infected and about 40 million people chronically disabled. Being the third leading parasitic cause of morbidity and disability worldwide, this disease not only affects physical health, but also causes social isolation and stigma (Fenwick, 2012). According to WHO Global Health Estimates 2015, nearly 2 million Disease Adjusted Life Years (DALYs) were reported due to LF (World Health Organization, 2016). Luckily, no direct mortality caused by LF has been observed, but the disease causes a huge economic, psychological, and social impact. Significant disfigurement of body parts including the genitals (hydrocele) and extremities (lymphoedema and elephantiasis) lead to pain and severe debility which ultimately lead to incapacitation for work and permanent disability. Moreover, a recent report indicated the economic loss caused by LF in India alone reaches an estimated US$ 1.5 billion due to temporary and permanent disabilities (Ramaiah et al., 2000, 2003; Ramaiah, 2013).

Table 1. Global prevalence and distribution of major human nematode infections

Disease	Major etiologic agent	Global prevalence	Regions of highest prevalence
Filarial nematodes			
Lymphatic Filariasis	*Wuchereria bancrofti, Brugia malayi*	120 million (Michael et al., 2009)	Southeast Asia, India and sub-Saharan Africa
Onchocerciasis (river blindness)	*Onchocerca volvulus*	18 million (Ndyomugyenyi, 1998)	Sub-Saharan Africa
Loiasis	*Loa loa*	13 million (Boussinesq, 2007)	Sub-Saharan Africa
Soil-transmitted nematodes			
Ascariasis	*Ascariasis lumbricoides* (roundworm)	807 million (Berthony. et al.,2006)	Regions of Asia, Africa and Latin America
Trichuriasis	*Trichuris trichiura* (whipworm)	604 million (Berthony. et al.,2006)	Regions of Asia, Africa and Latin America
Hookworm	*Necator americanus, Ancylostoma duodenale*	576 million (Berthony. et al.,2006)	Rural poverty regions of Asia, Africa and Latin America
Strongyloidiasis	*Strongyloides stercoralis* (thread worm)	3-100 million (cdc.gov/parasites/ strongyloides/epi.html)	Rural poverty regions of Asia, Africa and Latin America

- **table adapted from:** (Hotez et al., 2008)

1.3.1 Endemicity of LF

W. bancrofii is the most common cause of LF, responsible for nearly 90% of cases due to its wide geographical distribution and its presence in Africa, Asia, the Caribbean, Latin America

and many islands of the Western and South Pacific Ocean. *B. malayi* makes up the remaining 10% of the cases and is geographically restricted to South-West India, China, Indonesia, Malaysia, Korea, the Philippines and Vietnam. In a few regions the distribution of *B. malayi* and *W. bancrofti* overlap. *B. timori* is a recently identified causative agent for LF and is confined to the Lesser Sunda islands of Indonesia. Nonetheless, all these three species show high similarities in their life cycles. All three species are transmitted by mosquito vectors, namely *Aedes, Anopheles, Culex* and *Mansonia spp*. *Culex* mosquitoes are the main vector for LF in most urban and sub-urban areas, while *Anopheles* species and *Aedes* mosquitoes transmit LF in rural areas and in endemic islands of the Pacific region, respectively. According to the WHO Global Programme for Elimination of Lymphatic Filariasis (GPELF) (Ottesen, 1998), LF has been targeted for elimination as a public health problem by 2020 using intensive mass drug administration (MDA) programs with anti-filarial drugs. In spite of the strict implementation of MDA since 2000 only 20 of the 72 countries are now under post-MDA surveillance, whereas in the remaining countries the effective MDA regimen is still in under progress (Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO, 2017; Owusu et al., 2018).

1.3.2 Life cycle of the filarial parasites

The life cycle of all three filarial worm species includes mosquito and human stages. Humans are the definitive host and mosquitoes the intermediate host of Bancroftian and Brugian filariasis (Figure 2). Infection is initiated by the deposition of the infective stage larvae (L3) on the skin of a human host during a mosquito bite. The parasite larvae then pass through the skin puncture and reach the afferent lymphatic vessels. Although the exact number of L3 larvae required to successfully transmit the infection is not known, an average resident in an endemic area is exposed to 50 to 300 L3 larvae per year (Hati et al. 1989). After reaching the lymphatics and lymph nodes, the L3 larvae undergo two subsequent moulting stages, first to the L4 larval stage in 7 to 10 days, and before maturing into adult worms 4-6 weeks after L3 entry. Adult worms reside permanently in the lymphatic system and adult female parasites can be reproductively active for at least 5-8 years (Moreno and Geary, 2008; Genchi et al., 2012). The average length of male and female worms is 40 mm and 50 – 100 mm, respectively. Both male and female worms undergo sexual mating and females start producing approximately 50,000 microfilariae (Mf) per day (Nelson 1981), which then circulate in the peripheral blood circulation. Generally, Mf of *W. bancrofti* and *B. malayi* appear in large numbers in the peripheral circulation during night time, a phenomenon is called nocturnal periodicity.

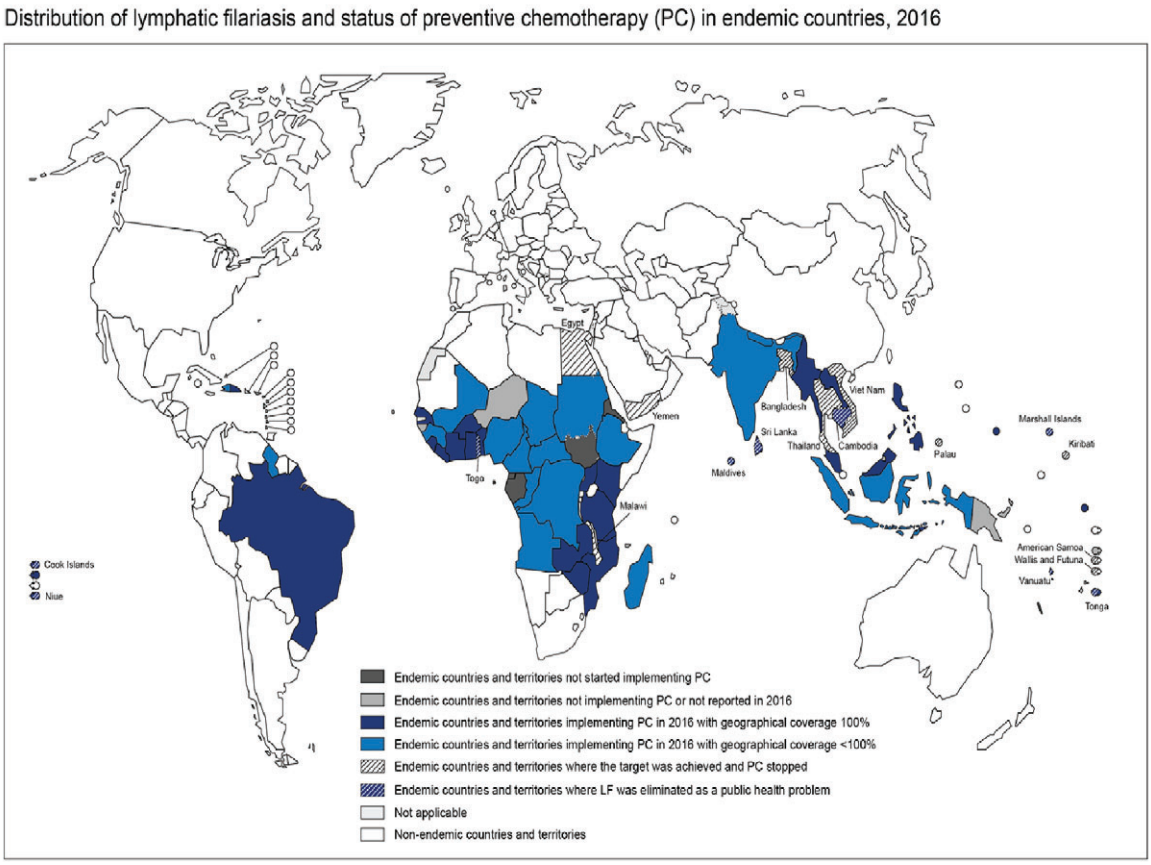

Figure 1. Areas endemic for lymphatic filariasis (in shades of blue) and status of MDA implementation (Map and data was adapted from WHO, status of LF in 2016, http://gamapserver.who.int/mapLibrary/Files/Maps/LF_2016.png)

The circulated Mf in the blood are then picked up by a mosquito during a subsequent blood meal, wherein they develop into L1, L2, and finally the infective stage L3 larvae thereby completing the transmission cycle of the parasite. In the mosquito, the Mf undergo the following three stages of development: (a) 1 to 2 hours after ingestion, the larvae come out of the sheath in which they are enclosed, called ex-sheathing of larvae, (b) development of L1-larvae: within 6 to 12 hours, the larvae enter the stomach wall of the mosquito and migrate into the thoracic muscles wherein they grow and develop to L1 larvae, (c) development of L2 larvae: The L1 larvae undergo further moulting as L2 larvae by growing in length, (d) development of infective L3 stage larvae: In this step, the highly active and motile form of infective larvae (long, thin form) are found in any part of the insect and when they reach the proboscis of the mosquito, they are ready to be transmitted to a new host to initiate the filarial life cycle.

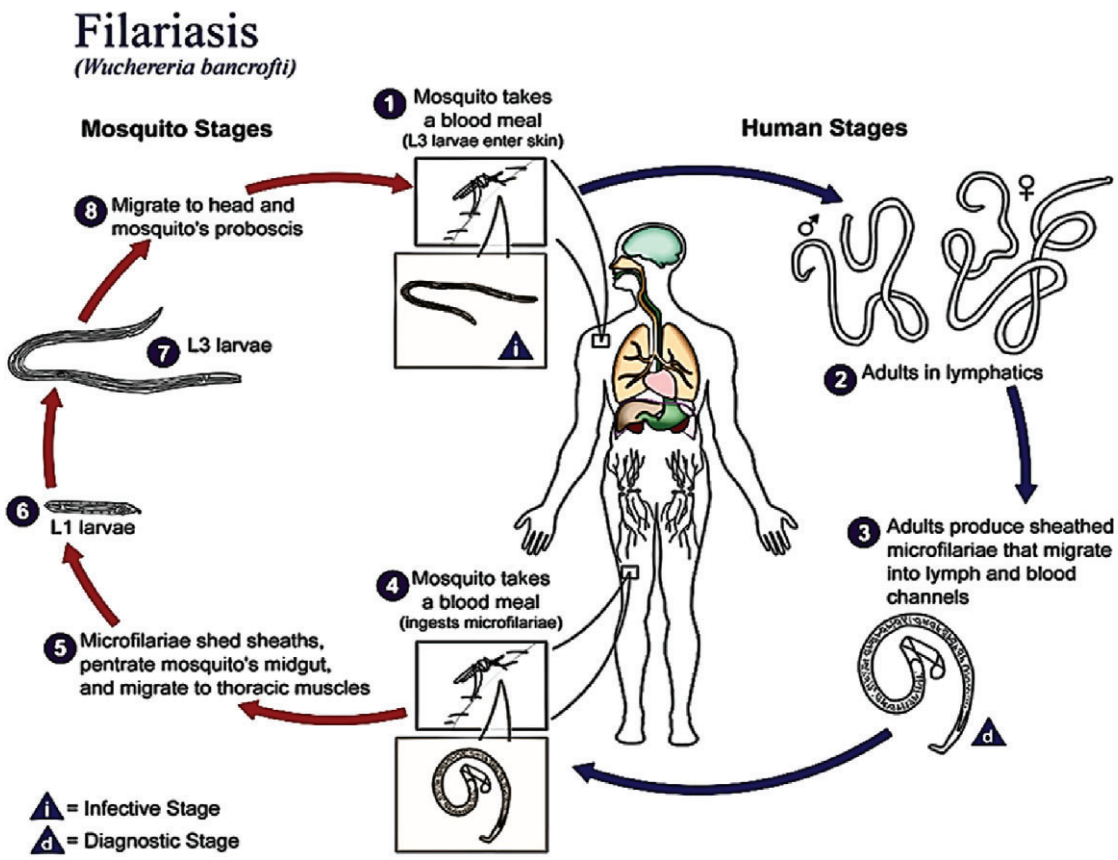

Figure 2. Life cycle of filarial parasites. The left part of the panel shows the life cycle stages of filarial worms inside the mosquito, and in the right part displays the life cycle stages in the human host (picture adapted from CDC: http://www.cdc.gov/parasites/lymphaticfilariasis/biology_w_bancrofti.html)

1.3.3 Clinical spectrum of LF

The clinical manifestations of LF range from asymptomatic to acute and chronic infections such as lymphangitis, lymphadenitis, and elephantiasis of genitals, breasts, legs or arms. Although the infection is acquired during childhood and causes hidden damage to the lymphatics, LF-induced symptoms such as hydrocele (or) lymphoedema occur mostly with advanced age. The important factors associated with disease manifestations in human hosts are age and sex. Generally, in endemic areas, all age groups are susceptible to infection as infants aged less than 6 months were also diagnosed with filarial infection. Infection rates increase with age though filarial disease appears only in a small proportion of infected individuals (Tisch et al., 2001; Babu and Nutman, 2012). Sex is also considered to be an important factor as Mf rates are higher in men compared to women in most endemic areas (Pfarr et al., 2009). Moreover, the chronicity of LF is also more commonly seen in men when

compared to women (Surendran et al., 1996; Pfarr et al., 2009; Babu and Nutman, 2014). The disease manifestations of LF have been classified as follows:

Endemic normals: In endemic areas, some percentage of the population does not have Mf in their blood circulation although they have been exposed to the same degree of infection as the rest of the population. This putatively immune group is commonly seen with the absence of microfilaremia; high molecular-weight antigen; and the absence of circulating IgG4, an antibody isotype associated with active infection.

Asymptomatic infection (sub-clinical): In most endemic areas, a substantial proportion of the population is asymptomatic, and they may remain without symptoms for years or in some cases for a life-time. These asymptomatically infected individuals do not exhibit any external signs or symptoms even though they are positive for Mf presence in their blood circulation (Taylor et al., 2010).

Acute and chronic infection: These patients have recurrent incidents of local inflammation which mainly involve the skin, lymph nodes and lymphatic vessels in the extremities, as well as breasts and genitals. Due to the impairment of host immunity and lymphatic damage, these patients have acute immune responses against the parasites. Acute adenolymphangitis is one such example, characterized by lymphatic inflammation (lymphangitis and lymphadenitis) with high fever and transient local edema. Sometimes lymph vessel dilation is also seen when adult worms are still alive. The features of this acute condition are mostly caused by immune responses against dead worms or bacteria as secondary infection. Although the mechanisms of acute manifestations remain largely unexplained, in most cases acute inflammatory reactions can function as a root cause for developing a chronic condition such as chronic lymphedema (tissue swelling) or elephantiasis (skin/tissue thickening). These patients are commonly observed with lymphedema or elephantiasis of limbs and hydrocele (fluid accumulation) (Taylor et al., 2010).

Chronic LF: Usually, the chronic stage is postulated to develop 10-15 years after initial infection, because chronic inflammation occurs predominantly due to spontaneous worm death (or) due to the effect of antifilarial drugs (Pfarr et al., 2009; Taylor et al., 2010). Death of adult worms can lead to lymphangitis and lymphadenitis with localised pain and swelling. Moreover, the amount of exposure to infection and magnitude of host immunity to infection influences the risk of developing chronic disease. Typically, chronic stage patients are mostly diagnosed with fibrosis and/or obstruction of lymphatic vessels causing permanent structural changes. The main clinical features of chronic bancroftian filariasis are hydrocele,

lymphedema, elephantiasis and chyluria. Usually, hydrocele patients have significant accumulation of edematous fluid in the cavity of the tunica vaginalis testis. Likewise, chronic epididymitis patients have inflammation of the epididymis and patients who are diagnosed with funiculitis will have inflammation of the spermatic cord. Chyluria patients have excretion of chyle, a milky white fluid in the urinary tract though prevalence of this condition is very low. (McNulty et al., 2013; Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO, 2017).

Lymphedema is one of the commonly seen conditions among LF patients; the severity of lymphedema and progression to elephantiasis can be classified by the following grades: Grade I, mostly pitting edema which is spontaneously reversible; Grade II, mostly non-pitting edema not spontaneously reversible; and finally Grade III, patients with elephantiasis and discolouration of the skin.

1.3.4 Diagnosis for LF

Generally, the diagnosis of LF can be made by detecting Mf in the peripheral blood circulation (blood taken predominantly at night time due to nocturnal periodicity of *W. bancrofti*), but detecting L3, L4 and adult worms is very difficult which due to their limited presence only at lymphatic vessels or sinuses of lymph nodes. Based on the involved vector and the filarial species, Mf periodicity is either diurnal (during the day) or nocturnal (during the night). Additionally, circulating filarial worm antigen can be detected by using either enzyme-linked immunosorbent assay (ELISA) or a rapid-format immunochromatographic card test. Molecular xenomonitoring of parasites in pools of mosquitoes is yet another approach used to assess LF prevalence. Recently, the Luciferase immunoprecipitation system (LIPS assay) is used to detect the presence of LF as it showed considerable sensitivity of 76% for *W. bancrofti*.

1.3.5 Treatment for LF

As per WHO, the approved drugs that are used to control and treat filariasis include diethylcarbamazine, ivermectin, and albendazole (Ottesen, 1998; Ramaiah and Ottesen, 2014). Combination of antiparasitic drugs such as albendazole either with diethylcarbamazine (DEC) or ivermectin are also commonly used to treat LF as per the Global Programme to Eliminate Lymphatic Filariasis (GPELF). Furthermore, in many endemic areas, antibiotics like doxycycline are also used along with the drugs that are mentioned above to treat *Wolbachia* endosymbionts whereby the filarial worms serve as its host. Generally, microfilaricides display destructive properties against the microfilariae. Thereby, the treatment regimens recommended by GPELF to eliminate LF comprises annual MDA of antifilarial drugs to achieve interruption of microfilarial transmission targeting the people living in endemic areas,

and morbidity management and disability prevention to prevent and alleviate the suffering of affected individuals. GPELF is currently active in 53 of 73 countries that are endemic for LF. The implementation of MDA was done with the support and cooperation of national and local health officials, non-governmental organisations (NGOs), communities and most importantly with an active coordination by donors. More than five MDA rounds had given to lower the levels of infection where drug coverage was poor or where transmission was particularly intense. After the launch of GPELF in 2000, the annual coverage of MDA has expanded from 3 million people in 12 countries to 6.7 billion treatments delivered to 850 million people at least once. Twenty (28%) of 73 endemic countries are now under post-MDA surveillance to demonstrate that elimination has been achieved. MDA played an important role in primary prevention by decreasing and reducing transmission rates in populations at risk. The implementation of MDA is still active in India and the MDA coverage lead to reduced number of microfilaremic individuals, ultimately resulting in lowered asymptomatically infected individuals (Mf presence in blood). On the other hand, the outcomes of these drugs that are used as part of the MDA treatment regimen by the WHO have reported no or little effect in killing adult worms due to modest macrofilaricidal properties. Considering the current scenario and the drugs available for the treatment of LF, future research should strongly aim to discover new and more effective improved therapy (Krentel et al., 2013; Ramaiah and Ottesen, 2014).

1.4 Host immune responses during filarial infection

The exact role of host immunity in reducing inflammation and preventing disability during filarial infections on the one hand and attacking circulating microfilariae and adult worms on the other hand is still not completely understood and requires more research in the direction towards both innate and adaptive immune responses as these two domains are considered to be important in regulating the outcome of LF. Usually, filariasis patients exhibit local as well as systemic inflammation that involves various immune cell types and regulatory molecules in both the early and chronic phases of infection (Taylor et al., 2010; Specht et al., 2012). Numerous cell types are involved in the immune responses against filarial infection and most of these cells play a vital role in disease related pathology. The most important of these immune cell types which are known to be important during filarial infections are discussed in the following sections and also shown as an overview in Figure 3.

1.4.1 Monocytes

Monocytes are formed in the bone marrow from a common myeloid progenitor that is shared with neutrophils, and are then released into the peripheral blood circulation. About 5-10% of human peripheral-blood leukocytes are mature monocytes which are heterogeneous in

nature. Generally, monocytes migrate from blood vessels within 2-3 days and develop into macrophages in the tissues. Differentiated macrophages contribute to host defence, tissue remodelling and repair. Normally, monocytes are recruited within 8-12 hours during infection and inflammation, and based on the stimulus they can differentiate into macrophages of a particular phenotype. Although, we know that monocytes serve as precursors for macrophages they themselves have their own function including phagocytosis and cytokine production. There are at least three types of monocytes categorised in human blood based on their phenotypic receptors. The subsets of monocytes are classified based on the expression of large amounts of CD14 (receptor for LPS). However, the differential expression of CD14 and CD16 (which is also known as FcγRIII) divides the monocytes into the following three subsets: (i) classical monocytes (CD14++ CD16-), (ii) intermediate monocytes (CD14++ CD16+), and (iii) non-classical monocytes (CD14+ CD16++) (Gordon and Taylor, 2005).

In asymptomatic filarial infection, monocytes exhibit the characteristic features of an alternative activation phenotype as they produce varied immune regulatory molecules such as arginase-1 (Arg-1), resistin, mannose receptor C type 1 (MRC-1), macrophage galactose type C lectin (MGL), and chemokine ligand 18 (CCL18). Moreover, asymptomatically infected patients also exhibit the induction of a monocyte phenotype that partly resembles the alternatively activated state after stimulation with live filarial parasites (Babu et al., 2009). Interestingly, patent lymphatic filariasis patients have impaired monocyte and CD4+ T cell function, reflected by their inability to produce inflammatory cytokines in response to activating stimuli (Babu et al., 2005; Semnani et al., 2006), while elevated levels of IL-10 are observed in asymptomatic patients (Babu et al., 2009; O'Regan et al., 2014). Recently, we showed that monocytes from asymptomatically infected individuals harbouring *B. malayi* microfilariae upregulate programmed-death ligand 1 (PD-L1), IL-10 and IL-8. *In vitro* experiments using human monocytes and macrophages from non-endemic healthy donors stimulated with microfilarial lysate (MF) also showed upregulated expression of PD-L1 and IL-10. Additionally, MF-stimulated human monocytes co-cultured with autologous CD4+ T cells effectively suppress CD4+ T cell proliferation and function. (O'Regan et al., 2014). Furthermore, monocytes from asymptomatically infected filarial patients have reduced ability to produce IL-1β in response to LPS compared with monocytes from endemic normal (Sasisekhar et al., 2005). Overall, this strongly suggests the role of an immunomodulatory protein within microfilariae that can effectively contribute to the outcome of LF infections.

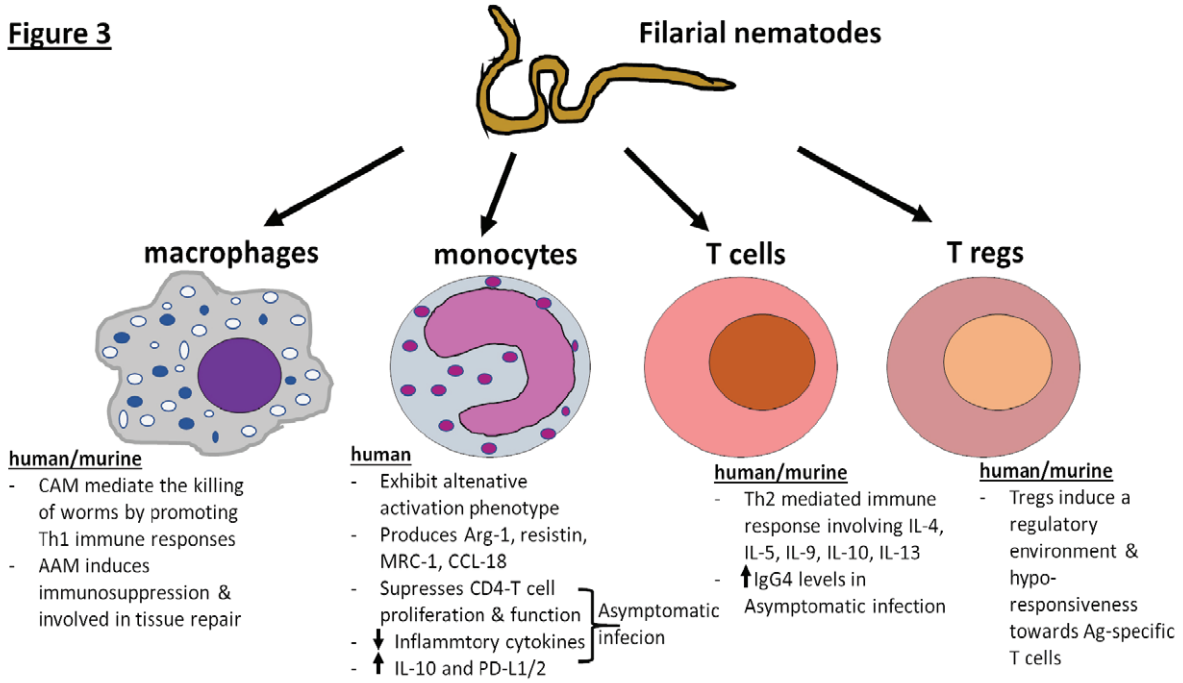

Figure 3. The involvement of immune cells and their responses during filarial nematode infections.

1.4.2 Macrophages

Tissue macrophages are specialized immune cells that have a broad role in keeping organisms healthy by maintaining tissue homeostasis, clearing senescent cells, as well as mediating wound healing and repair mechanisms after inflammation (Gordon and Taylor, 2005). Inflammatory monocytes are recruited and differentiate into macrophages at the site of an inflammatory lesion, with two types of macrophages observed during infections: (i) classically activated macrophages (CAM or M1 macrophage), which can be induced *in vitro* by culture of macrophages with IFNγ and LPS (which induces TNF production); (ii), alternatively activated macrophages (AAM or M2 macrophage) which result from culture with IL-4 or IL-13. As was clearly demonstrated in LF patients, a balance between CAMs and AAMs is essential and directly related to severity of the disease (Anuradha Rajamanickam, 2013; Wynn et al., 2013). In filarial infections, CAMs mediate the killing of worms and induce Th1 responses, ultimately leading to pathology like elephantiasis, whereas AAMs control these responses, so it is very important to maintain the equilibrium between these two macrophage subsets (Allen and Loke, 2001).

CAMs play a vital role in controlling acute infections due to their phagocytic activity and promotion of Th1 immune responses, thereby inducing secretion of pro-inflammatory cytokines such as TNFα, IL-6 and nitric oxide (NO) (Allen and Loke, 2001; Mosser, 2003). In contrast, AAMs are considered to be important in host immunity against the chronic phase of

infections due to their capacity to induce immunosuppression and their involvement in tissue repair (Mosser, 2003; Gordon and Martinez, 2010). AAMs induce immunosuppression by increased IL-10 production, and thus ultimately facilitate wound healing, angiogenesis, and extra cellular matrix deposition (Rodríguez-Sosa et al., 2002; Mosser, 2003; Gordon and Martinez, 2010). Recently, many studies used murine models of filarial infections to characterise macrophage populations precisely during filariasis. Along with *in vitro* data, these studies suggest that macrophages play a prominent role in immune evasion strategies for the parasites, affecting both effector and suppressor cells with the help of CAMs and AAMs respectively. Previously, it was shown that macrophages which were activated by IFNγ kill *B. malayi* Mf by releasing NO (Taylor et al., 1996). In contrast, another study demonstrated that IFNγ and NO were not essential for immune mediated clearance of *B. malayi* Mf (Gray and Lawrence, 2002). Interestingly, another study reported that excretory/secretory (E/S) products from live adult *B. malayi* worms induce AAMs, after intraperitoneal implantation in mice. (Allen and Macdonald, 1998). In parallel, there was a study demonstrating diminished macrophage activation in a jird infection of *B. pahangi;* differentiated macrophages did not become activated even after exposure to adult worms and also failed to produce NO after LPS stimulation (Nasarre et al., 1998).

1.4.3 CD4+ T cell and its subsets

Commonly, helminth parasites are known to be effective immune regulators due to their ability to suppress, divert, and convert the host immune response to the benefit of the pathogen. Helminths induce a distinct immune response profile unlike microbial pathogens. Immunity against helminths, including filarial nematode infections, is largely mediated by Th2 type immune response, whereas Th1 type immune responses are generated mostly in bacterial infections (Allen and Maizels, 2011). Th1 immunity characterized by IFNγ production was evolved to control innate anti-microbial pathways, while Th2 immunity controls the host defence system and mainly involved in innate tissue repair process during metazoan parasite infection (Allen and Maizels, 1996, 2011; McSorley and Maizels, 2012). Generally, naïve CD4+ T cells can differentiated into T helper (Th1), T helper (Th2), T helper 17 (Th17), T helper (Th9) and regulatory T cells (Treg) (Luckheeram et al., 2012). Th2 type mediated immune responses involve the cytokines interleukin-3 (IL-3), IL-4, IL-5, IL-9, IL-10 and IL-13, the antibody isotypes IgG1, IgG4 and IgE, and expanded populations of eosinophils, basophils, mast cells and alternatively activated macrophages (Akdis et al., 2011; Taylor et al., 2012; McSorley et al., 2013). The profile of antibody isotypes, especially the relative levels of IgG4 and IgE, appears to be the most important factor determining susceptibility to and protection from helminth infection, respectively (Allen and Maizels, 2011; McSorley and Maizels, 2012). Moreover, the production of IgG4 compared to IgE levels reached

extraordinarily high levels against the filarial antigens in the asymptomatically infected individuals (Babu and Nutman, 2014).

It has been well demonstrated that both type 1 and type 2 immune responses are involved during filarial infection, as elimination of worms and development of pathology were seen in parallel within the host (Babu and Nutman, 2014). Also it was noted that naïve T cells were differentiated into Th2 type cells after stimulation with live L3 of *Litomosomoides sigmodontis* (Specht et al., 2012). Previously, it was reported that IL-5 controls worm survival, both at the larval and adult stages, while IL-4 impairs the development of patent filarial infection (Volkmann et al., 2001, 2003; Le Goff et al., 2002). However, lack of IFN-γ production lead to an increased worm burden during *L. sigmodontis* infection (Saeftel et al., 2001; Hoerauf et al., 2005). Also in human filarial infections, numerous studies have been published and demonstrate the role of CD4+ T cells in host immune response during filarial infections (Pearlman et al., 1995; Hoerauf et al., 2005; van der Werf et al., 2013). Most of the studies on immunity to parasitic filariasis in humans has been demonstrated using endemic normal individuals who live in a filaria-endemic region (Babayan et al., 2012). Interestingly, endemic normal individuals have a mixed Th1/Th2 response and also a strong CD4+ T cell proliferative responses. These endemic normals typically have elevated levels of the Th2 cytokine IL-5 and the Th1 cytokine IFNγ (Turaga et al., 2000) in response to PBMCs that are stimulated with both L3 larvae and antigens from adult male of *Onchocerca volvulus*, a tissue-dwelling filarial species (Turaga et al., 2000). Moreover, *W. bancrofti* infected individuals demonstrated a strong CD4+ T cell proliferation and produce significant levels of IFN-γ in *B. malayi* adult antigen stimulated PBMC of endemic normals when compared to asymptomatic individuals (Sasisekhar et al., 2005).

1.4.4 Regulatory T cells (Treg)

Generally, the longevity of parasites within the host, and subsequent chronicity of infection is directly related to the ability of worms to suppress host immune responses. Moreover, the inflammatory responses against the parasite lead to the release of many proteins that augment tissue damage. Regulatory T cells (Treg) function as part of this regulatory machinery to effectively suppress both Th1 and Th2 immune response, thereby increasing the worm load and controlling tissue damage (Hoerauf et al., 2005). Markers for Treg cells include fork head box P3 (FoxP3), a transcription factor, CD25 and gluco-corticoid-induced TNFR family related genes (GITR) (Hoerauf et al., 2005; Adalid-Peralta et al., 2011). There are two types of Treg cells based on their origins: thymic or natural Treg (nTreg) and inducible Treg (iTreg). In human filarial infection, Tregs induce a regulatory environment and hypo-responsiveness

towards antigen-specific T cells with the reduction of Th1 and Th2 immune response (Taylor et al., 2005). Previously, it was also shown that Treg cells from asymptomatically infected individuals can effectively suppress Th2 cell proliferation and reduce expression of Th2 cytokines in response to BmA (Wammes et al., 2012). On the other hand, studies from murine filariasis, especially with *L. sigmodontis* infection, demonstrate that removal of Treg cells can increase the immune response against the parasite and also prevent prolonged infection (Taylor et al., 2005; Dittrich et al., 2008).

1.5 Immunomodulatory products derived from helminths

Generally, the capacity to modulate the immune system by helminth-derived molecules determines the longevity of helminth parasites in the mammalian host. Immunomodulatory helminth-derived products induce a microenvironment within the host which is beneficial to the parasite. These molecules include protease inhibitors and homologues of mammalian cytokines and chemokines that induce a modified Th2-type immune response (Hartmann et al., 1997; Dainichi et al., 2001; Manoury et al., 2001; Schönemeyer et al., 2001; Pfaff et al., 2002; Schnoeller et al., 2008). *Bm-tgh-1* and *Bm-tgh-2* are two transforming growth factors of (TGF)-β homologues found in Brugian species, in which *Bm-tgh-2* showed significant influence on T-reg development by binding to the TGF receptor (Gomez-Escobar et al., 1998, 2000). Another study demonstrated the immunomodulatory potential of excretory/secretory products (ES) from *H. polygyrus* which contains TGF-β like activity, inducing Foxp3 expression in naïve T cells and thus ultimately helping to maintain the worm burden to induce chronic infection (Grainger et al., 2010). Moreover, the macrophage migration inhibitory factor (MIF) homologues from *B. malayi* prevent random migration of cells especially human monocyte behaviour. Besides this, MIF from helminths showed direct chemotactic effects on human monocytes and also exhibit modified Th2-type responses (Zang, 2002).

Previously, it has been demonstrated that ES-62, an excretory-secretory product of the rodent filarial nematode *Acanthocheilonema viteae,* induces tyrosine phosphorylation of glycoproteins in murine macrophages to inhibit the production of IL-12 if the cells are in the presence of lipopolysaccharide (LPS) and IFN-γ (Helen S Goodridge et al., 2005). Likewise, omega-1, a hepatotoxic ribonuclease, is a glycoprotein present in Schistosome soluble egg antigen (SEA) induces Th2 dependent responses and also exhibits the ability of this helminth to modulate host immune responses (Everts et al., 2009). Cysteine and serine protease inhibitors are considered another family of important immunomodulatory helminth-derived molecules due to their high immunomodulatory potential (McSorley and Maizels, 2012;

McSorley et al., 2013). Filarial cystatins contribute to a large part to this thesis and will be discussed further in depth in the next section.

1.5.1 Cysteine proteases

Proteases (or) peptidases are a large family of peptide bond cleaving hydrolases and are further categorized as endopeptidases (proteinases) and exopeptidases based on cleaving site. Based on the reactive groups, which are present at the active site involved in catalysis, these enzymes are further classified as serine peptidases, cysteine proteases, aspartic endopeptidases, and metallopeptidases. Among these, serine peptidase was studied extensively and well characterized (Otto and Schirmeister, 1997). Cysteine proteases are found almost in all species, in viruses, bacteria, fungi and plants as well as in parasites and mammals.

1.5.2 Cysteine protease inhibitors (or) Cystatins

Cystatins, a family of cysteine protease inhibitors (CPIs), the best-characterized protease inhibitors secreted by helminths, have immunomodulatory potential. Mostly, cystatins are known to regulate cysteine protease functions such as protein catabolism, antigen processing, inflammation, dystrophy and metastasis in viruses, bacteria, protozoa, fungi, plants and mammals. In general, all CPIs only inhibit cysteine proteases, including the exopeptidase cathepsin C. Moreover, cysteine proteases can be inactivated by competitive, noncovalent, reversible inhibition. In contrast to serine protease inhibitors, the CPIs usually form complexes with cysteine protease whose active sites have reacted with a thiol blocking reagent. Mammalian cysteine proteases are essential for effective processing and presentation of antigen on MHC class II to induce an appropriate adaptive T cell response. Cystatins play a vital role in regulating these pathways by inhibiting cysteine proteases (cathepsins and aspartyl endopeptidases) which are required for antigen processing and presentation and thus inhibiting T cell activation (Grzonka et al., 2001; Hartmann and Lucius, 2003; Gregory and Maizels, 2008; Kopitar-Jerala, 2012).

1.5.3 The Cystatin Superfamily

Based on sequence homologies, cysteine protease inhibitors (CPIs) from mammals are classified into the following three families: the **stefins**, **cystatins** and **kininogens**. (i) Stefins are a family of proteins lacking disulfide bridges, carbohydrate residues, and signal peptides. Stefins are intracellular proteins and have a molecular weight of about 11 kDa (approximately, 100 amino acids). (ii) Secondly, the cystatins have molecular weights of 12 to 13 kDa (about 110-120 amino acids) and have two disulphide loops at the C terminal, but lack carbohydrate

residues. Cystatin C, D, S, SN and SA belong to the cystatin family. The presence of Cystatin C is not only restricted to the extracellular space, but also found in some other cells such as in cortical neurons, in pancreatic islet cells, in the thyroid glands and in parotid glands. Cystatin S, SN and SA have similar primary structures. Cystatin S and D were isolated from human saliva. Cystatin S also found in different salivary glands, in serum, in gall, in tear fluid, urine, pancreas and bronchi. (iii) Finally, kininogens are the third member of the cystatin super family and have high molecular weight protein ranging from 50 kDa to 120 kDa. Usually, the kininogens are large plasma proteins which possess typical characteristics for extracellular proteins including carbohydrate residues, signal peptides and disulfide bridges(Hartmann and Lucius, 2003; Gregory and Maizels, 2008).

1.5.4 Filarial cystatins

Filarial parasites employ strong immunoregulatory effects on the host immune system and exhibit profound immune evasion strategies such as immunosuppression, immunological tolerance and modification of stereotypical Th2 responses. Generally, immunosuppression is characterized by immunoregulatory cytokine induced suppression of immune responses, whereas immunological tolerance was estimated based on the state of immune tolerance in effector T cells. Instead, the switching of antibody isotype into IgG4 in humans, and also an induction of alternatively activated macrophages occurs in during modified Th2 responses. Filarial E/S products which have the ability to modulate host immune function are considered to be important immune evasion strategies employed by these parasites. Filarial cystatin is one such molecule studied widely due to its dual action of inhibiting nematode cystatin as well as host proteases. So far, there were a few studies demonstrating the involvement of filarial cystatin in the regulation of parasite proteases during molting, and also inhibiting cysteine proteases which are required for host APC antigen processing and presentation thereby leading to diminished T-cell priming. Moreover, previous results from our lab also elucidated the ability of filarial cystatin to induce immunosuppressive IL-10 and nitric-oxide producing regulatory macrophages, which can result in the direct impairment of T-cell proliferation (Hartmann and Lucius, 2003; McSorley and Maizels, 2012).

Cystatin from filarial rodent parasites, for example AvCystatin/AV17 of *A. viteae,* downregulates T cell responses and showed an effective interference with MAP kinase signalling to induce IL-10 (Hartmann et al., 1997; Klotz et al., 2011). Results from our own group revealed that AvCystatin induced production of IL-10 in murine macrophages was tyrosine kinase sensitive, and dependent on activation of both MAPK (Klotz et al., 2011). Similarly, Ls-cystatin from *L. sigmodontis* effectively reduces antigen-specific proliferation of spleen cells (Pfaff et al., 2002). On the other hand, onchocystatin from the human filarial

nematode *O. volvulus* suppresses antigen-specific proliferation of PBMCs and induces IL-10 from stimulated PBMCs. Additionally, it was demonstrated that onchocystatin reduces the expression of MHC class II molecules and CD86 on human monocytes (Schönemeyer et al., 2001).

Importantly, three cysteine protease inhibitors have been characterized in the human filarial nematode *Brugia malayi* (*B. malayi*)*:* Bm-CPI-1, Bm-CPI-2 and Bm-CPI-3. Among these, Bm-CPI-2 is expressed in all life cycle stages of *B. malayi*, whereas the expression of Bm-CPI-1 and -3 are limited to the L2 and L3 stages and are mainly involved in the mosquito vector necessary for transmission of the parasite (Manoury et al., 2001). Moreover, Bm-CPI-2 is noteworthy due to its ability to block antigen presentation via MHC class II by interfering with asparginyl endopeptidase (Manoury et al., 2001; Gregory and Maizels, 2008). Previously, we showed that human monocytes and murine macrophages are target cells of filarial cystatin both *in vitro* and *in vivo* (Schönemeyer et al., 2001; Schnoeller et al., 2008). Previously, we demonstrated that monocytes from asymptomatic individuals harbouring microfilaria from *B. malayi* in addition to *in-vitro* experiments using microfilarial lysate (MF) stimulated human monocytes and macrophages from non-endemic healthy donors, upregulate IL-10, IL-8 and programmed-death ligand 1 (PD-L1). Moreover, the MF-stimulated monocytes were able to suppress CD4+ T cell proliferation and functions significantly (O'Regan et al., 2014). The previously understood immunomodulatory potential of Bm-CPI-2 from various studies, and the excessive secretion of Bm-CPI-2 in *B. malayi* microfilaria (Bennuru et al., 2011; Morris et al., 2015) prompted us to further study the actual contribution of this protein to immunomodulate human immune cells. Therefore, we aim to investigate the exact role (or) potential contribution of Bm-CPI-2 in MF-induced immunomodulation of human monocytes and macrophages. (Figure 4). Additionally, we were interested in identifying the key host signalling events (illustrated in Figure 5) exploited by Bm-CPI-2 and MF to modulate these myeloid cell types (Venugopal et al., 2017).

Figure 4.

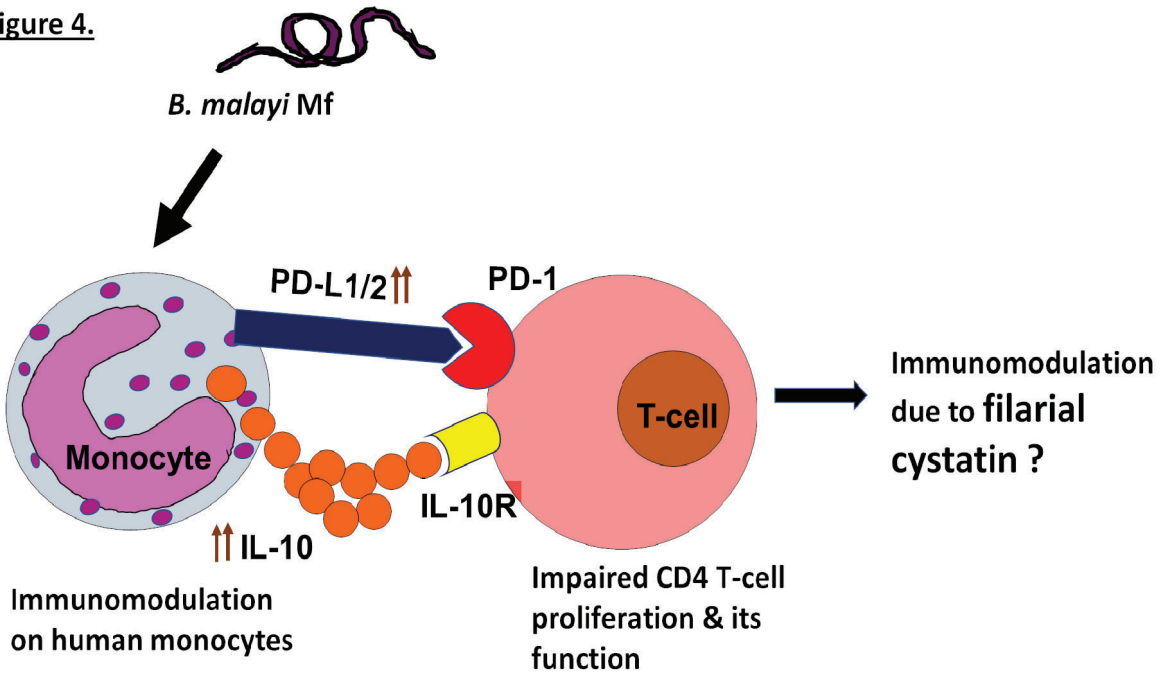

Figure 4. Diagram depicts the ability of *B. malayi* Mf lysate to immunomodulate human monocytes and suggesting a possible role of filarial cystatin from *B. malayi* Mf lysate.

Figure 5. MAPK inhibitors pathways

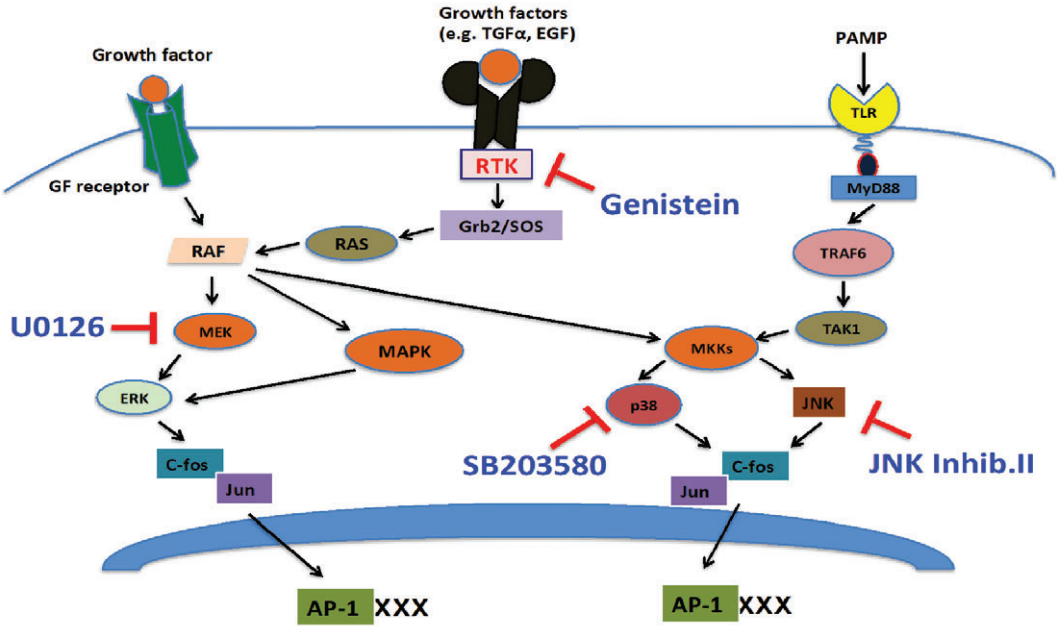

Figure 5. Identification of possible host signalling events exploited by rBm-CPI-2 and *B. malayi* Mf lysate to induce IL-10 in human monocytes and macrophages.

1.6 Immunogenetics of lymphatic filariasis (LF)

As mentioned, LF is an important neglected tropical disease responsible for causing morbidity throughout the tropics and sub-tropics. LF is generally considered to be a complex, multifactorial disease which encompasses a wide spectrum of clinical manifestations associated with both genetic and environmental factors, which might influence disease outcome (Choi et al., 2001, 2003; Nutman, 2013). Although the actual cause of heterogeneity in filarial infection and disease is not fully understood yet, current available epidemiologic evidence suggests that the contribution of a genetic component is inevitable to the susceptibility and the clinical outcomes of LF (Hotez et al., 2008; Babayan et al., 2012; Johnston et al., 2014). Therefore, to explore the fundamental insights of biology, evolution and pathophysiology of humans and other species, understanding the correlation between genetic variation and biological function are necessary (Babayan et al., 2012). Recently, many genetic association studies have reported with filarial infection especially after the completion of human genome and the generation of draft sequence for *B. malayi* with the help of HAPMAP(Risch, 2000; Ghedin et al., 2007). This provided ample opportunities to examine the role of allelic variation which are believed to be important determinants of disease development. Single nucleotide polymorphism (SNP) is one of the common types of genetic variation that is highly abundant, and is estimated to occur at 1 out of every 1,000 bases in the human genome (Balding and Balding, 2006; Johnson, 2009; Clarke et al., 2011; Ha et al., 2014; Oh and Deasy, 2014). The different consequences at the phenotypic level depend on where the SNP occurs. SNPs in the coding regions of genes are regularly analyzed for diagnostic purposes as these SNPs can alter the function or structure of the encoded proteins, which are necessary and sufficient to cause clinical differences in most of the diseases. Genome linkage scans and candidate gene analysis have been used to identify genetic risk factors in many infectious and autoimmune diseases. However, the candidate gene approach was applied in most of the gene association studies due to its potential in identifying the genetic variations very precisely, and thus notify the risk for disease outcome (Syvänen, 2001; Johnson, 2009).

The second aim of this thesis was to identify the potential genetic polymorphisms of host immune genes in filarial infections by using a candidate gene approach, as this could provide new insights into the mechanisms underlying a spectrum of disease outcomes. So far, only a few immune related gene polymorphisms were tested and identified as a potential polymorphic association with LF. These include SNPs from the following genes: Chitotriosidase (CHIT 1), TLR2 and TLR4, VEGF-A, VEGF-C and IL-13 (Choi et al., 2001; Hoerauf et al., 2002; Debrah et al., 2007). Hence, we were interested in investigating the possible associations of SNPs from regulatory genes which are known to be involved in host immune responses during filarial

infections; importantly SNPs from IL-10 and PD-1 pathway genes. IL-10 is an anti-inflammatory cytokine which plays an important role regulating inflammatory disease like allergies and autoimmune disorders. Generally, IL-10 is secreted by myeloid cells, B cells and lymphocytes, and suppresses the activation of T helper cells and reduces the production of pro-inflammatory cytokines. IL-10 receptor complex is comprised of both IL-10 receptor (IL-10RA) and IL-10 receptor 2 (IL-10RB). Programmed cell death 1 (PD-1) is an immune inhibitory receptor mainly expressed on activated T cells, B cells, and myeloid cells. There are two ligands for PD-1, which includes programmed cell death ligand– 1 and 2 (PD-L1 and PD-L2). These ligands are expressed mainly on antigen presenting cells (APCs) such as macrophages and dendritic cells and upon interaction of PD-1 with its ligands, lead to inhibition of T cell receptor mediated lymphocyte proliferation and cytokine secretion, as well as blockade of CD28-mediated co-stimulation. Numerous gene association reports were published and indicated associations of IL-10 and PD-1 pathway gene variants, but predominantly with infectious diseases (e.g. extra-pulmonary tuberculosis, influenza, leprosy and malaria); autoimmune diseases (e.g. ankylosing spondylitis, diabetes mellitus, systemic lupus erythematosus and ulcerative colitis); as well as asthma and cancer. Thus far, no gene association studies have been reported on SNPs from IL-10 and PD-1 pathway genes with disease outcome in LF.

1.7 Aims and objectives:

The first aim of this thesis was to identify the exact contribution of *Brugia malayi* cystatin on immunomodulation on human monocytes and macrophages

The specific objectives of this aim were:

1. To determine the potential of recombinant BmCPI-2 to induce a regulatory phenotype in human monocytes and macrophages
2. To determine if IL-10 production by microfilarial lysate (Mf) in human monocytes and macrophages is dependent on the presence of filarial cystatin
3. To investigate the signalling events (importantly, the involvement of MEK and p38 signalling) exploited by rBmCPI-2 and Mf lysate to induce IL-10 and IL-8 in human monocytes and macrophages.

The second aim of this thesis was to understand the implication of SNP in candidate genes on the susceptibility and/or clinical outcome of LF.

2. Original articles

2.1 Differential immunomodulation in human monocytes versus macrophages by filarial cystatin – https://doi.org/10.1371/journal.pone.0188138

(PLoS One. 2017 Nov 15;12(11):e0188138. doi: 10.1371/journal.pone.0188138. eCollection 2017).

2.2 Association of a PD-L2 Gene Polymorphism with Chronic Lymphatic Filariasis in a South Indian Cohort - https://doi.org/10.4269/ajtmh.18-0731

(Am J Trop Med Hyg. 2019 Feb;100(2):344-350. doi: 10.4269/ajtmh.18-0731).

PLOS ONE

RESEARCH ARTICLE

Differential immunomodulation in human monocytes versus macrophages by filarial cystatin

Gopinath Venugopal, Marion Mueller, Susanne Hartmann*, Svenja Steinfelder

Institute of Immunology, Centre for Infection Medicine, Freie Universität Berlin, Berlin, Germany

* susanne.hartmann@fu-berlin.de

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Abstract

Parasitic nematodes have evolved powerful immunomodulatory molecules to enable their survival in immunocompetent hosts by subverting immune responses and minimizing pathological processes. One filarial molecule known to counteract host immune responses by inducing IL-10 and regulatory macrophages in mice is filarial cystatin. During a patent filarial infection monocytes encounter microfilariae in the blood, an event that occurs in asymptomatically infected filariasis patients that are immunologically hyporeactive. The microfilarial larval stage was formerly shown to induce human regulatory monocytes and macrophages. Thus, here we aim was to determine how filarial cystatin of the human pathogenic filaria *Brugia malayi* (BmCPI-2) contributes to immune hyporesponsiveness in human monocytes and macrophages elicited by microfilaria. For this purpose, filarial cystatin was depleted from microfilarial lysate (Mf). Detecting the immunomodulatory potential of cystatin-depleted Mf revealed that IL-10, but not IL-8 and IL-6 induction in monocytes and macrophages is dependent on the presence of cystatin. In addition, the Mf-induced expression of the regulatory surface markers PD-L1 and PD-L2 in human monocytes, but not in macrophages, is dependent on cystatin. While Mf-treated monocytes result in decreased $CD4^+$ T-cell proliferation in a co-culture assay, stimulation of T-cells with human monocytes treated with cystatin-depleted Mf lead to a restoration of $CD4^+$ T-cell proliferation. Moreover, IL-10 induction by cystatin within Mf was dependent on p38 and ERK in macrophages, but independent of the ERK pathway in monocytes. These findings indicate that filarial nematodes differentially trigger and exploit various signaling pathways to induce immunomodulation in different myeloid cell subsets.

Introduction

Helminths play a central role in causing infections among communities living in tropical and subtropical regions, and about one third of the world population is infected with one or more parasitic helminths[1]. The two major classes of helminths are nematodes and platyhelminths, both of which contain causative agents of a variety of acute debilitating diseases and chronic conditions[2]. In general, helminth infections are well characterized by strong Th2 immune responses, and are able to down-regulate host immune responses[1,3,4].

Lymphatic filariasis (LF) is one of seventeen Neglected Tropical Diseases (NTDs) classified by the World Health Organization (WHO)[5], and is the third leading parasitic cause of morbidity and disability worldwide. LF is normally associated with varied clinical outcomes and according to WHO Global Health Estimates (GHE), in 2015 alone LF contributed close to 2 million Disease Adjusted Life Years (DALYs)[6]. The causative agents of human LF are three species of parasitic nematodes, namely *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. Over 120 million people around the world are infected with either of these parasites and about 40 million people suffer from disfigurement and incapacitation for work as a result of LF-induced symptoms like lymphedema, elephantiasis or hydrocele[7]. The lifecycle of these filarial worm species comprises of both mosquito (*Aedes*, *Anopheles*, *Culex* or *Mansonia* spp.) and human stages. Infection with the parasite is initiated when an infected mosquito deposits the infective larvae stage on the skin of a human host during a blood meal. The parasite larvae then reach the afferent lymphatic vessels, where they are able to undergo two moulting stages and eventually mature to adult worms. After mating, females start producing microfilariae, which are then able to circulate in the peripheral blood of the human host. During a subsequent blood meal the mosquito vector is therefore able to pick up circulating blood microfilariae, thus initiating the development of the infective L3 stage larvae and completing the transmission cycle of the parasite.

The host-parasite interaction in LF often results in a chronic infection associated with a wide clinical spectrum, characterized by functional dysregulation of both the innate and the adaptive immune responses[8]. Three of the key clinical manifestations of this disease include asymptomatic infection, despite the presence of the larval blood stages (microfilariae) caused by hyporesponsiveness towards the parasite, chronic pathology with hydrocele, or elephantiasis, with or without active infection displaying a Th1/Th17-biased immune response, and endemic normality, where the host is putatively immune against the parasite.

The longevity of helminth parasites in the mammalian host is primarily determined by their capacity to modulate the immune system. Immunomodulatory helminth-derived products such as protease inhibitors[9–14] and homologues of mammalian cytokines and chemokines[15–18] induce a modified Th2-type immune response, which creates a beneficial microenvironment for the parasite[19,20]. Cystatins, a family of cysteine protease inhibitors, are known to regulate cysteine protease functions such as protein catabolism, antigen processing, inflammation, dystrophy and metastasis in viruses, bacteria, protozoa, fungi, plants and mammals [21–24]. Helminth cystatins are highly expressed protease inhibitors and they have been shown to possess strong immunomodulatory properties. They have a dual function, due to their ability to inhibit both parasite and host cysteine proteases. Cystatins are reversible competitive inhibitors of cysteine proteases and control protein processing and turnover, thereby inhibiting antigen processing[10,11], leading to diminished T cell priming. Secondly, they induce the immunosuppressive cytokine IL-10[9] and nitric oxide-producing regulatory macrophages[25], which can result in the inhibition of T cell proliferation, as shown in previous studies on cystatins from the filarial rodent parasites *Acanthocheilonema viteae* [9] and *Litomosoides sigmodontis* [14], and the human filarial nematode *Onchocerca volvulus* [12].

We could show in previous studies that monocytes and macrophages are target cells of filarial cystatins *in vitro* and *in vivo*[12,13]. Moreover, monocytes from asymptomatic individuals harbouring microfilariae from *Brugia malayi* upregulate programmed-death ligand 1 (PD-L1), IL-10 and IL-8, which is recapitulated in experiments using human monocytes and macrophages from non-endemic healthy donors stimulated with microfilarial lysate (Mf) *in vitro*. Importantly, macrophages differentiated in the presence of Mf lysate respond poorly to LPS, and Mf-stimulated monocytes are able to suppress $CD4^+$ T cell functions[26]. This is in line with the finding that patent lymphatic filariasis patients have impaired monocyte and $CD4^+$ T cell function, reflected by their inability to produce inflammatory cytokines in response to

activating stimuli[27,28], while elevated levels of IL-10 are observed in asymptomatic patients [26,29]. The anti-inflammatory cytokine IL-10 plays a major role in regulating inflammatory diseases such as allergies and autoimmune disorders[30], while IL-8 is an important chemokine with a key role in the pathogenesis of filarial diseases[31] and it displays elevated levels in patients with and without circulating microfilariae[32].

There are three known cystatins from the human filarial nematode *Brugia malayi* (BmCPI-1, -2 and -3). Among these three types, BmCPI-2 is expressed both in the vector and in the mammalian life stages while BmCPI-1 and -3 are exclusively expressed in the mosquito stages of the parasite [10,33]. Most importantly, BmCPI-2 is released in greater amounts by *Brugia malayi* microfilaria compared to adults stages[34]. This prompted us to investigate the immunomodulatory potential of BmCPI-2 on human monocytes and macrophages. Furthermore, we tried to determine the contribution of *Brugia malayi* cystatin in Mf-induced immunomodulation of human monocytes and macrophages, and to further demonstrate the key host signalling events used by BmCPI-2 and Mf to modulate these host cell types. In the present study, we demonstrate that the expression of IL-10 by Mf-stimulated monocytes and macrophages is dependent on cystatins. Moreover, we show that IL-10 expression in monocytes, but not in macrophages, is independent of ERK signalling, suggesting different pathways of IL-10 induction in these cell populations.

Materials and methods

Human blood samples

Human blood cells were derived from the German Red Cross and all experiments were approved by the ethics committee of the Charite University, Berlin, Germany.

Filarial lysate preparation

Live *Brugia malayi* microfilariae were kindly donated by the NIAID/NIH Filariasis Research Reagent Resource Center in Athens, Georgia, USA. The worms were initially washed twice in RPMI medium containing 1% glucose, 200 U/ml penicillin and 200 μg/ml streptomycin, and incubated with the same medium at 37˚C and 5% CO_2 for 24h, followed by washing in phosphate buffered saline (PBS). To prepare the filarial lysate, microfilariae worms were homogenised directly in a glass homogeniser and ultrasonicated on ice at an intensity of 10% for 3 min. The homogenate was centrifuged at 12,000 rpm at 4˚C for 10 min and sterile filtered through a 0.22 μm filter. Protein concentration from the filtered homogenate was determined by using the Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL, USA), as per the manufacturer's guidelines.

Immunoprecipitation of *Brugia malayi* cystatin

An immunoprecipitation technique was performed to deplete *Brugia malayi* cystatin from protein mixtures of microfilarial lysate (Mf) by using rabbit polyclonal antibodies raised against BmCPI-2, recombinantly expressed in *E. coli*. The assay was performed using an immunoprecipitation kit from Novex (Thermo Fisher Scientific, Waltham, MA, USA), as per the manufacturer's guidelines. Briefly, 50 μl of protein G-coupled Dynabeads1 were added to the buffer containing anti-BmCPI-2 antibodies (1:1000). After 10 min incubation, the supernatant was removed and beads were washed twice. Then, 100–150 μl of Mf was added to the beads. After 10–15 minutes incubation, the beads were removed, cystatin-depleted Mf was collected and the protein concentration was determined using the Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL, USA), as per the manufacturer's guidelines.

Cloning of the BmCPI-2 sequence into the LEXSY expression system and purification of BmCPI-2

The nucleotide sequence of BmCPI-2 was amplified from *Brugia malayi* microfilaria cDNA using specific primers (forward: 5′-gTCgACgCTTTgATTCATCgACgAg-3′, reverse: 5'-ggTACCTACTgACgAgAgTACCTTTg-3') including restriction sites for cloning the sequence into the pLEXSY-sat2 plasmid of the LEXSY con2 Expression Kit (Jena Bioscience GmbH, Jena, Germany). The amplified sequence did not include the coding region for the specific signal peptide of BmCPI-2. Cloning procedures followed the manufacturer's instructions and resulted in a monoclonal LEXSY cell strain expressing and secreting BmCPI-2 with a C-terminal hexa-histidine tag into the culture medium. Purification of the protein was performed via affinity chromatography using HisTrap™excel columns and the ÄKTA™ pure chromatography system (GE Healthcare Bio-Science AB, Uppsala, Sweden) with a non-denaturing protocol, with imidazole as a competitive eluent. The purified protein was dialysed against PBS and sterile filtered. The protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA).

Immunoblot analysis

SDS-PAGE was performed in 12% separating gel with 6% stacking gel. 15 μg of protein from each sample was applied per lane. Proteins were transferred to a nitrocellulose membrane for immunodetection. After blocking with 5% non-fat dry milk in TBS-Tween, membranes were incubated with rabbit anti-BmCPI-2 (1:50,000), followed by incubation with horseradish peroxidase conjugated anti-rabbit IgG (1:15,000) (Cell signalling, Danvers, MA, USA). Signals were detected by a chemiluminescence reaction using the ECL kit (Amersham, GE Healthcare Europe, GmBH). All bands were visualized by an enhanced chemiluminscence system (PEQLAB, Erlangen, Germany).

Protein detection by silver staining

Silver staining was performed to detect proteins separated in SDS-PAGE by using Thermo Scientific Pierce Silver Stain kit (Thermo scientific, Rockford, IL, USA) using the manufacturer's protocol. Briefly, 15 μg of protein from each sample was applied per lane in SDS-PAGE. After running the gel for 1 h, the gel was washed twice with ultrapure water and then fixed twice by adding 30% ethanol and 10% acetic acid for 15 min each. After 30 min of fixation, the gel was washed twice, first with 10% ethanol then with ultrapure water. After the washing step, the gel was sensitized quickly for 1 min using silver stain sensitizer then washed twice with ultrapure water. The gel was then incubated for 30 min with a silver stain enhancer and washed briefly with ultrapure water. The gel was developed using silver stain developer solution and the development procedure was stopped immediately after the desired band intensity was achieved using 5% acetic acid. After the addition of stop solution, the gel was washed briefly with ultrapure water and incubated for 10 min at room temperature.

Isolation of $CD14^+$ monocytes and differentiation protocol for monocyte-derived macrophages from human PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by density gradient centrifugation using Pancoll human (PAN Biotech, Aidenbach, Bayern, Germany). To positively select for $CD14^+$ monocytes, 200 μl of anti-CD14 beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) were added to 5-10x10^8 PBMCs for 20 min and cells were separated by an autoMACS classic (Miltenyi Biotec, Bergisch-Gladbach, Germany) using the program

'Possel'. Macrophages were generated *in vitro* by culturing $CD14^+$ monocytes in complete RPMI containing 10 ng/ml human M-CSF in 6-well cell culture plates at a cell concentration of 0.33 x 10^6/ml and a density of 0.1x10^6/cm^2 for 6 days, at 37˚C and 5% CO_2.

Cell culture and stimulation of monocytes and macrophages

The $CD14^+$ monocytes and monocyte-derived macrophages were stimulated with parasite material or recombinant BmCPI-2 and kept for 24 h at 37˚C and 5% CO_2. The supernatant was collected after 24 h and stored at -20˚C for further analysis. In experiments including inhibitors, the inhibitors were added to the cell culture 60 min before stimulation. The following inhibitors were used in our study: p38 inhibitor (SB203580), and MEK1/2 inhibitor (U0126) (both from Calbiochem, Merck, Darmstadt, Germany).

Flow cytometry

Monocytes and macrophages were analysed for surface expression of PD-L1 and PD-L2. Cells were treated with FcR blocking reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany), stained with fixable viability dye eFluor 780 (eBiosciences, San Diego, USA) and additionally stained for the following markers: anti-274-PE (clone MIH1), anti-273-PE (clone MIH18). Fixed cells were acquired using the FACS Canto II (BD, Franklin Lakes, USA) and analysed using FlowJo, version 10.2 (Tree Star, Ashland USA).

Co-culture of $CD4^+$ T cells and monocytes

To perform the co-culture experiments for a functional assay of T cell proliferation, $CD4^+$ T cells were isolated from PBMCs using the $CD4^+$ T cell microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions and sorted on an auto-MACS classic (Miltenyi Biotec, Bergisch-Gladbach, Germany) using the program 'Possel'. The autologous $CD4^+$ T cells were stained for CFSE and rested in complete RPMI for 24 h at 37º C and 5% CO_2.

Monocytes were left unstimulated or stimulated with 20 μg/ml of Mf or cystatin-depleted Mf lysate for 24 h in a 5% CO_2 incubator at 37º C in 24 well plates. 5x10^5 CFSE-labelled $CD4^+$ T cells were co-cultured with 1x10^5 stimulated monocytes in 96 well flat bottom plates in the presence of 2 μg/ml soluble anti-CD3 (OKT3, eBioscience, San Diego, USA). After 5 days, the cells were washed and stained with fixable viability dye eFluor 780 (eBioscience, San Diego, USA) and anti-CD4-PerCP (clone RPA-T4, BioLegend, San Diego, USA). Fixed cells were acquired using the FACS Canto II (BD, Franklin Lakes, USA) and analysed using FlowJo, version 10.2 (Tree Star, AshlandUSA).

Cytokine detection by ELISA

IL-10, IL-8, IL-6 and TNFα proteins were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA). IL-12p40 was also measured using an ELISA kit (BioLegend, San Diego, CA, USA). All samples were measured in duplicates. Absorbance was read at 450nm with background wavelength subtracted at 570nm using a Synergy HT plate reader (BioTek, Winooski, VT, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, Inc., CA, USA). Comparisons between groups were performed using the Wilcoxon signed rank test.

Results

BmCPI-2 from *Brugia malayi* induces IL-10, IL-8, IL-6 and PD-L1/L2

We have previously shown that *Brugia malayi* microfilarial lysate (Mf) induces a regulatory subset of human monocytes expressing IL-10 and PD-L1 leading to inhibition of $CD4^+$ T-cell proliferation and cytokine secretion[26]. In the present study, we hypothesized that the observed immunomodulation seen might be due to the presence of *Brugia malayi* cystatin, as the immunomodulatory potential of cystatin has been demonstrated in murine macrophages [9,14]. First, to determine the potential of BmCPI-2 to induce a regulatory phenotype in human monocytes and macrophages, we tested if BmCPI-2 is able to induce inflammatory and anti-inflammatory cytokines and programmed death-ligands in human monocytes and macrophages of healthy blood donors in a similar fashion as Mf[26]. To this end, we used recombinant BmCPI-2 (rBmCPI-2) that was expressed in a eukaryotic protein expression system. rBmCPI-2 stimulated monocytes to secrete IL-10, IL-8 and IL-6 in a dose-dependent manner, while IL-12p40 and TNFα were not induced (**Fig 1A)**. Likewise, rBmCPI-2 induced IL-10, IL-8 and IL-6 in a similar fashion in human macrophages (**Fig 1B**). The observed increase in IL-12p40 production in human macrophages was not significant between unstimulated cells and cells stimulated with 20 μg/ml of rBmCPI-2 (Fig 1B). Moreover, rBmCPI-2-stimulated monocytes and macrophages showed significant up-regulation of PD-L1 and PD-L2 (**Fig 2**).

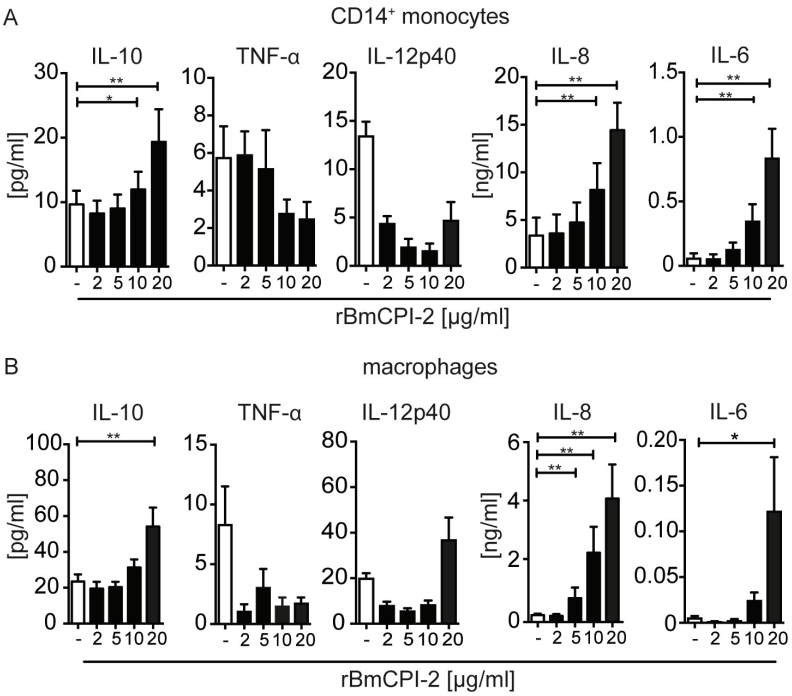

Fig 1. *Brugia malayi* cystatin-2 (rBmCPI-2) induces IL-10, IL-8 and IL-6 in human monocytes and macrophages. Human monocytes (A) and macrophages (B) were stimulated with different concentrations [μg/ml] of recombinant BmCPI-2 for 24h. Cytokines in the supernatant were detected by ELISA. Data are from 12 donors and shown as mean ±SEM. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test in reference to the unstimulated control. *p<0.05, **p<0.01.

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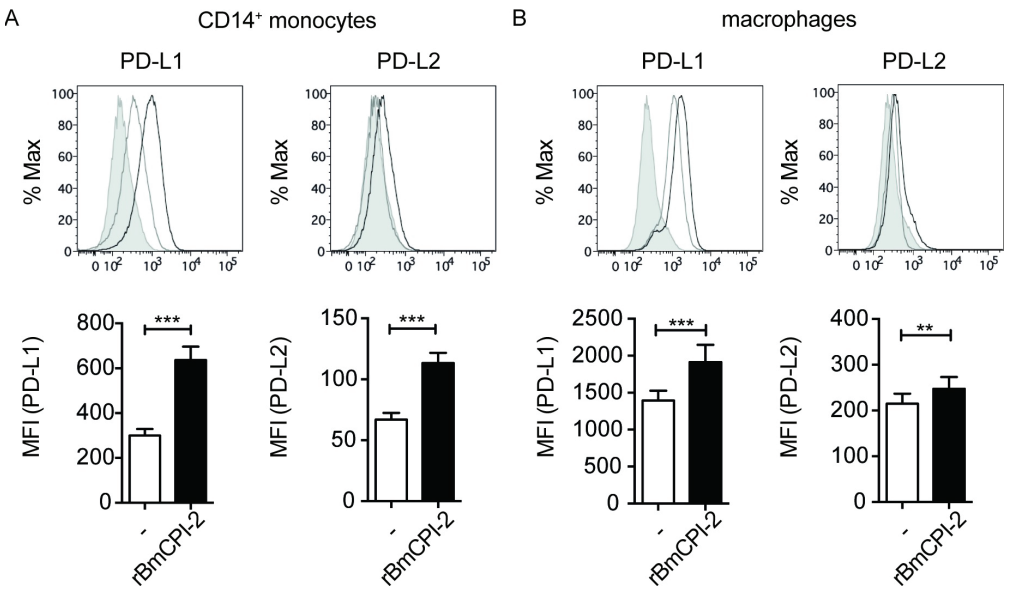

Fig 2. rBmCPI-2 induces expression of PD-L1 and PD-L2 on human monocytes and macrophages. Monocytes (A) or macrophages (B) were left unstimulated or stimulated for 24 h with 20 μg/ml of recombinant BmCPI-2 (n = 12). Surface expressions of PD-L1 and PD-L2 were measured by flow cytometry. Histograms in the upper panel show representative plots of unstained (grey tinted), unstimulated (grey line) and stimulated cells (black line). Bar graphs in the lower panel show the mean fluorescence intensity (MFI). All data are represented as mean ±SEM. P values were calculated using Wilcoxon signed-rank test. **p<0.01, ***p<0.001.

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Induction of IL-10 and PD-L1 and PD-L2 in human monocytes by *Brugia malayi* microfilarial lysate is dependent on cystatins

To determine if IL-10 production by microfilarial lysate (Mf) in human monocytes and macrophages is dependent on the presence of filarial cystatin, we depleted all three forms of cystatins from Mf (termed here ΔMf) by immunoprecipitation using polyclonal antibodies raised against recombinant BmCPI-2. BmCPI-2 was indicated by a black arrow in Fig 3A. Successful depletion of *Brugia malayi* cystatins from Mf was obtained without altering the overall protein composition of Mf (**Fig 3A and 3B**). Monocytes and macrophages stimulated with ΔMf did no longer produce significant amounts of IL-10, similar to unstimulated controls, in contrast to non-cystatin-depleted-Mf-stimulated monocytes (**Fig 3C)** and macrophages (**Fig 3D**), which induced significant amounts of IL-10. These data suggest that IL-10 induction by Mf is strictly cystatin-dependent in monocytes and macrophages.

On the other hand, Mf-induced IL-8 was not strictly dependent on cystatins, since IL-8 levels were lower but not significantly different in ΔMf- versus Mf-stimulated monocytes and macrophages. Similarly, Mf-induced IL-6 in monocytes was not dependent on cystatins, whereas in macrophages Mf did not induce IL-6 (Fig 3C and 3D). Notably, our results show that Mf-induced expression of PD-L1 and PD-L2 in monocytes is partially dependent on cystatins (**Fig 4A**). On the other hand, expression of PD-L1 in human macrophages was not dependent on cystatins (**Fig 4B**). Next, we employed a functional assay for $CD4^+$ T-cell proliferation using autologous monocytes treated with Mf or ΔMf and co-cultured both cell types with anti-CD3. We observed a significant reduction of proliferation when T-cells were co-

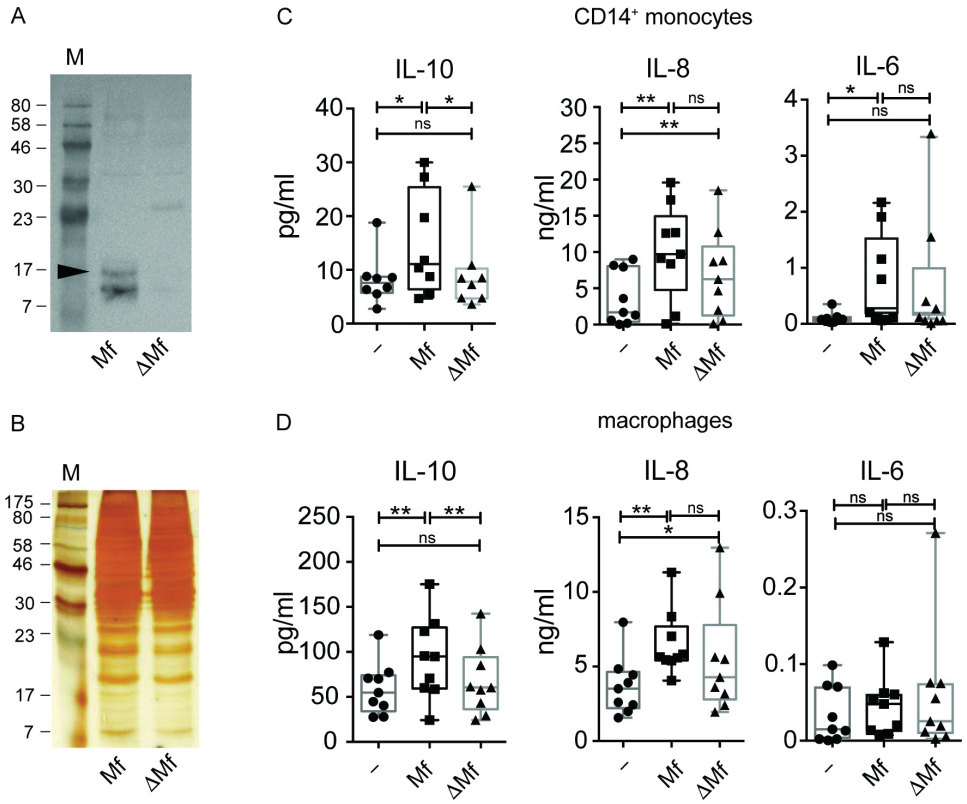

Fig 3. *Brugia malayi* microfilaria lysate induced IL-10 is dependent on the presence of filarial cystatins. Western Blot (A) and Silver gel (B) showing *Brugia malayi* microfilaria lysate (Mf) and microfilaria lysate depleted of filarial cystatins (ΔMf). The black arrow indicates BmCPI-2. C) Human monocytes, and D) monocyte-derived macrophages were stimulated with 20 μg/ml of Mf or ΔMf for 24 h. Cytokines in the supernatant were detected by ELISA. Data are from 8–9 donors and shown as mean ±SEM. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test. *p<0.05, **p<0.01.

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cultured with Mf-treated monocytes compared to unstimulated monocytes as described before [26]. Strikingly, when T-cells were co-cultured with ΔMf-stimulated monocytes proliferation was not significantly altered with respect to co-culture with unstimulated monocytes. Moreover, ΔMf-stimulated monocytes significantly restored T-cell proliferation compared to Mf-stimulated monocytes (Fig 4C).

Hence, IL-10 induction by Mf, but not IL-8 or IL-6, appears dependent on cystatins in both myeloid cell types. Furthermore, PD-L1 and PD-L2 expression of monocytes but not macrophages is dependent on cystatins. Most importantly, cystatins mediate the inhibitory potential of monocytes, which leads to reduced $CD4^+$ T-cell proliferation.

Differential induction of IL-10 by rBmCPI-2-stimulated myeloid cell population

Previous studies indicate that the increased production of IL-10 in murine macrophages by cystatin from the rodent filarial nematode *Acanthocheilonema viteae* was critically dependent on two mitogen-activated protein kinases, ERK and p38[35]. Nevertheless, the immune

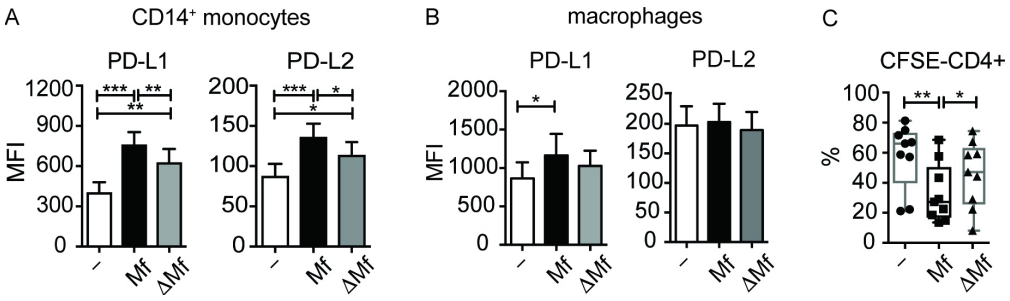

Fig 4. Mf-induced PD-L1 and PD-L2 on monocytes is dependent on the presence of filarial cystatins. A) Human monocytes, and B) monocyte-derived macrophages were stimulated with 20 μg/ml of Mf or ΔMf for 24 h. Surface expression of PD-L1 and PD-L2 was measured by flow cytometry. Bar graphs show the mean fluorescence intensity (MFI). Data are from 16 (monocytes) and 9 (macrophages) donors, respectively, and shown as mean ±SEM. C) Flow cytometric analysis of $CD4^+$ T cells. $5x10^5$ CFSE-labelled $CD4^+$ T cells were co-cultured for 5 days with $1x10^5$ monocytes left either unstimulated or stimulated for 24 h with 20 μg/ml Mf or 20 μg/ml ΔMf. Graph shows the percentage of CD4+ T cells that divided. Data are from 9 donors and shown as mean ±SEM. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test. *p<0.05, **p<0.01, ***p<0.001.

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signalling events exploited by human filarial cystatin to induce IL-10 in human monocytes and macrophages is still elusive. This directed us to investigate the signalling events activated or altered by rBmCPI-2 and Mf, and leading to IL-10 and IL-8 production in human monocytes and macrophages. To this end, we used various concentrations of inhibitor molecules known to be involved in the regulation of IL-10 production[35].

Firstly, we determined the role of the MEK-dependent signalling pathway to induce IL-10 and IL-8 in rBmCPI-2-stimulated human monocytes and macrophages. Monocytes (**Fig 5A**) and macrophages (**Fig 5B**) were incubated with rBmCPI-2 for 24 h in the presence of various concentrations U0126, which is a specific inhibitor of MAP kinase kinases, MEK1 and MEK2, and thus ERK activation. Notably, rBmCPI-2-induced-IL-10 production was independent of ERK signalling, as MEK inhibition did not alter the production of IL-10 in human monocytes (**Fig 5A**), a finding also seen with Mf-stimulated monocytes (**S1 Fig**). In contrast, rBmCPI-2-induced IL-10 production in macrophages was dependent on ERK signalling, as we have observed a significant reduction of IL-10 levels in MEK inhibition (**Fig 5B**). Moreover, we

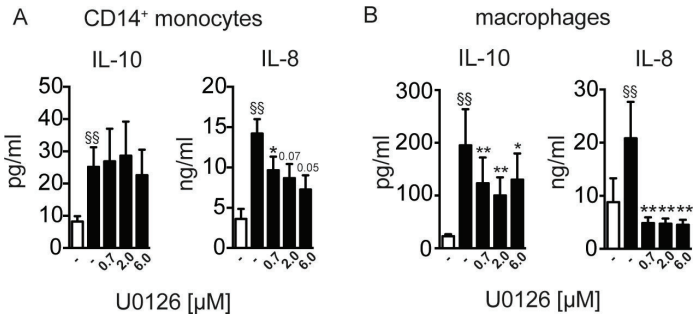

Fig 5. Induction of IL-10, but not IL-8 by rBmCPI-2 in human monocytes is independent of ERK. Human monocytes (A) and macrophages (B) were stimulated with 20 μg/ml of rBmCPI-2 for 24 h with various concentrations of MEK1/2 inhibitor. Cytokines in the supernatants were detected by ELISA. Data are from 7–9 donors and shown as mean ±SEM. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test. Values statistically different from the unstimulated control are depicted as § p<0.05 and §§ p<0.01. Values statistically different from rBmCPI-2-stimulated monocytes are depicted as * p<0.05 and **p<0.01.

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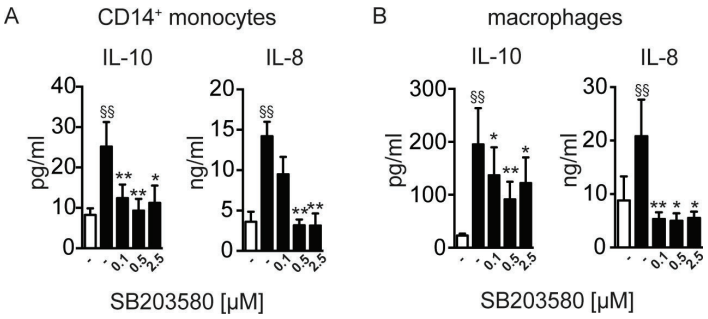

Fig 6. Induction of IL-10 and IL-8 by rBmCPI-2 in human monocytes and macrophages is dependent on p38. Human monocytes (A) and macrophages (B) were stimulated with 20 µg/ml of rBmCPI-2 for 24 h with various concentrations of p38 inhibitor. Cytokines in the supernatants were detected by ELISA. Data are from 7–9 donors and shown as mean ±SEM. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test. Values statistically different from the unstimulated control are depicted as § p<0.05 and §§ p<0.01. Values statistically different from BmCPI-2-stimulated monocytes are depicted as * p<0.05 and **p<0.01.

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observed a significant dose-dependent reduction of IL-8 levels in rBmCPI-2 (**Fig 5A**) stimulated monocytes and macrophages in the presence of the ERK inhibitor MEK1/2. This finding suggests that IL-10 induction by rBmCPI-2 and Mf is differentially regulated in human monocytes and macrophages.

Induction of IL-8 and IL-10 by rBmCPI-2-stimulated myeloid cell population is dependent on p38

We next investigated the possible involvement of the p38-signalling pathway triggered by rBmCPI-2 in inducing IL-10 and IL-8 production in both human monocytes and macrophages. Inhibition of signalling pathways dependent on p38 by addition of the inhibitor SB203580 resulted in a significant reduction of IL-10 and IL-8 in both rBmCPI-2 and Mf-stimulated monocytes and macrophages (**Fig 6A and 6B**, **S2 Fig**).

Discussion

The contribution of helminth-derived products towards the modulation of host immune responses has been addressed by several studies, and cystatin is one such secreted protein of filarial nematodes with strong immunomodulatory capacities[19,36]. With the present study we aimed to show the ability of *Brugia malayi* filarial cystatin to induce human monocytes and macrophages with a regulatory phenotype and function.

First, we demonstrated that the nematode immunomodulatory protein BmCPI-2 can significantly induce IL-10, IL-8 and IL-6, but not TNFα and IL-12p40 in human monocytes (**Fig 1A)** and macrophages (**Fig 1B**). Additionally, BmCPI-2 induced the expression of PD-L1 and PD-L2 on the surface of human monocytes and macrophages, suggesting a regulatory monocyte and macrophage phenotype *in vitro* (**Fig 2**). This is in line with our published data on human monocytes and macrophages stimulated with whole lysate of *Brugia malayi* microfilaria (Mf), where monocytes and macrophages develop a characteristic regulatory phenotype with high expression of IL-10 and PD-L1/L2, and inhibit $CD4^+$ T cell function[26]. Therefore, we then tested whether the observed immunomodulation seen in human monocytes and macrophages by Mf is due to the presence of cystatin. To that end we analysed the modulatory capacity of Mf that was depleted of cystatin (ΔMf). Interestingly, using polyclonal serum raised

against recombinantly expressed BmCPI-2, the protocol resulted in the depletion of all cystatins formerly detectable in Mf. Our results show that Mf-induced IL-10, but not IL-8 and IL-6 production in monocytes and macrophages is due to the presence of cystatin (**Fig 3C and 3D**). This is in agreement with previous studies on the ability of filarial cystatins to induce IL-10 production in murine macrophages[9,37] and human monocytes[12]. Additionally, expression of PD-L1 and PD-L2 in monocytes but not in macrophages is dependent on the presence of cystatin in Mf. Finally, both, the reduced expression of IL-10 and PD-L1/2 in monocytes, which is observed in Mf depleted of cystatins, might contribute to the observed restoration of $CD4^+$ T-cell proliferation **(Fig 4)**.

Recently, many studies have been published addressing the therapeutic potential of cystatin in inflammatory diseases due to its anti-inflammatory activity. The modulation of innate immune cells and the profound suppression of the host immune system due to a characteristic anti-inflammatory milieu with increased levels of IL-10 and transforming growth factor (TGF)-β in chronic helminth infections can protect against allergic diseases[38–40]. The importance of IL-10 and of macrophages in suppressing the allergic effects has been described in a murine model of ovalbumin-induced allergic airway hyper-reactivity and DSS-induced colitis applying treatment with filarial cystatin[13]. Moreover, filarial cystatin reduces Respiratory Syncytial Virus (RSV)-inducing immunopathology by inducing high levels of IL-10 production by $CD4^+$ T cells in the airways and lungs of mice[41]. Cystatin from the nematode parasite *Ascaris lumbricoides* reduces inflammation in a mouse model of DSS-induced colitis by increasing the expression of IL-10, TGFβ with simultaneous reduction of IL-6 and TNFα [42]. Collectively, the studies described here indicate that cystatins from various nematodes not only function as immunomodulators in the infection setting but also have potential as therapeutic agents for inflammatory diseases. Our results highlight the unique role of cystatin to induce IL-10 in human monocytes and macrophages stimulated with microfilarial products. Also cystatin-dependent expression of PD-L1 and PD-L2 in human monocytes suggests that cystatin from microfilariae is partially involved in the immune regulation process and thus could contribute to asymptomatic infection through PD-L1:PD-1 dependent suppression of $CD4^+$ T cell function[26,43–45]. Since circulating microfilariae in asymptomatically infected individuals will readily come into contact with blood monocytes, the modulation of this particular immune cell is vital for the parasite's immune evasion strategy.

Similar to our previously published results on Mf [26], stimulation of monocytes and macrophages with whole Mf resulted in the production of significantly higher levels of IL-8 compared to unstimulated controls. The significant elevation of IL-8 by Mf-stimulated human monocytes and macrophages, however, was not dependent on cystatin (**Fig 3C and 3D**). Thus, the effect by Mf could be attributed to the presence of other molecules in Mf that are also able to induce IL-8. So far there are only a few studies revealing the potential of filarial products to induce IL-8, such as lipoproteins from *Wolbachia*, an obligate intracellular symbiont of *Brugia malayi*, which was shown to induce IL-8 in a TLR2/6-dependent manner[46]. While infective L3 larvae are not able to induce IL-8 in dendritic cells (DC) [47] or epithelial cells[48], it is expressed in a co-culture of DC and keratinocytes[49]. In contrast to infective larvae, live Mf and excretory/secretory products of female worms can induce the expression of IL-8 in human DC[50] and monocytes[51]. Previously, and also in the present study, we could show significant elevation of IL-6 by Mf in monocytes only, but not in macrophages. Although we see an increased production of IL-6 in Mf-treated monocytes (Fig 3C), IL-6 was not significantly reduced in monocytes that were treated with ΔMf. This result suggests that other molecules from whole Mf lysate were responsible for the elevated levels of IL-6 by monocytes.

Furthermore, we studied the immune signalling events exploited by rBmCPI-2 leading to the production of IL-10 and IL-8 in human monocytes and macrophages. It has previously

been demonstrated that ES-62, an excretory-secretory product of the rodent filarial nematode *Acanthocheilonema viteae* induces tyrosine phosphorylation of glycoproteins in murine macrophages[52] and modulates the activation of MAP kinases (ERK, p38 and JNK), thereby regulating cytokine production[53,54]. A study from our own group revealed that IL-10 production in murine macrophages triggered by cystatin from *A. viteae* was tyrosine kinase sensitive, and depended on activation of both MAPK[35]. In addition, LPS-stimulated microglia of mice *in vitro* showed that increased IL-10 mRNA expressions by filarial cystatin was dependent on ERK signalling[55]. In agreement with this data, our results indicate a significant reduction of rBmCPI-2- or Mf-induced IL-10 in macrophages, suggesting roles for ERK and p38 in these cells. On the other hand, IL-10 induction by rBmCPI-2 or Mf in human monocytes was independent of ERK signalling, indicating a differential induction of IL-10 in this myeloid cell population. This suggests that filarial products are able to differentially exploit and/or trigger the signalling events in human monocytes and macrophages, leading to induction of IL-10. Moreover, while IL-10 was independent of the ERK pathway in human monocytes only, IL-8 production was dependent on both MAPK kinases (ERK and p38) in monocytes (**Figs 5A and 6A**) and macrophages (**Figs 5B and 6B**).

This is the first study addressing the contribution of filarial cystatin within microfilariae, which circulate in the blood, to induce IL-10 in monocytes. Overall, our results show that IL-10 and IL-8 induction by human filarial cystatin in human monocytes and macrophages is dependent on p38, while only IL-10 induction in macrophages is additionally dependent of ERK signalling. Moreover, Mf-induced IL-10 production, but not IL-8 or IL-6 induction in human monocytes and macrophages is dependent on cystatin, suggesting that while cystatin is sufficient and necessary for IL-10 induction, factors other than cystatin are responsible for IL-8 and IL-6.

Supporting information

S1 Fig. Induction of IL-10, but not IL-8 by microfilarial lysate (Mf) in human monocytes is independent of ERK. Human monocytes (A) and macrophages (B) were left unstimulated or stimulated with 20 μg/ml of Mf for 24 h with various concentrations of MEK1/2 inhibitor. Cytokines in the supernatants were detected by ELISA. Data are from 7–9 donors and shown as mean ±SEM. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test. Values statistically different from the unstimulated control are depicted as § $p<0.05$ and §§ $p<0.01$. Values statistically different from Mf-stimulated monocytes are depicted as * $p<0.05$ and $^{**}p<0.01$.
(TIF)

S2 Fig. Induction of IL-10 and IL-8 by Mf in human monocytes and macrophages is dependent on p38. Human monocytes (A) and macrophages (B) were left unstimulated or stimulated with 20 μg/ml of Mf for 24 h with various concentrations of p38 inhibitor. Cytokines in the supernatants were detected by ELISA. Data are from 7–9 donors and shown as mean ±SEM. Statistical analysis was done using the Wilcoxon matched-pairs signed rank test. Values statistically different from the unstimulated control are depicted as § $p<0.05$ and §§ $p<0.01$. Values statistically different from Mf-stimulated monocytes are depicted as * $p<0.05$ and $^{**}p<0.01$.
(TIF)

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Author Contributions

Conceptualization: Susanne Hartmann, Svenja Steinfelder.

Data curation: Gopinath Venugopal, Susanne Hartmann.

Formal analysis: Gopinath Venugopal, Svenja Steinfelder.

Funding acquisition: Susanne Hartmann.

Investigation: Gopinath Venugopal, Marion Mueller, Svenja Steinfelder.

Methodology: Gopinath Venugopal, Marion Mueller, Svenja Steinfelder.

Project administration: Susanne Hartmann, Svenja Steinfelder.

Resources: Susanne Hartmann.

Software: Susanne Hartmann.

Supervision: Susanne Hartmann, Svenja Steinfelder.

Validation: Susanne Hartmann, Svenja Steinfelder.

Visualization: Gopinath Venugopal, Svenja Steinfelder.

Writing – original draft: Gopinath Venugopal.

Writing – review & editing: Susanne Hartmann, Svenja Steinfelder.

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Association of a *PD-L2* Gene Polymorphism with Chronic Lymphatic Filariasis in a South Indian Cohort

Gopinath Venugopal,[1] Noëlle L. O'Regan,[1] Subash Babu,[2] Ralf R. Schumann,[3] Aparna Srikantam,[4] Roswitha Merle,[5] Susanne Hartmann,[1] and Svenja Steinfelder[1,6]*

[1]*Centre for Infection Medicine, Institute of Immunology, Freie Universita¨t Berlin, Berlin, Germany;* [2]*National Institutes of Health, National Institute of Research in Tuberculosis (NIRT), International Centre for Excellence in Research, Chennai, India;* [3]*Institute for Microbiology and Hygiene, Charite´–Universita¨tsmedizin Berlin, Berlin, Germany;* [4]*Blue Peter Public Health and Research Centre, LEPRA Society, Hyderabad, India;* [5]*Department of Veterinary Medicine, Institute for Veterinary Epidemiology and Biostatistics, Freie Universita¨t Berlin, Berlin, Germany;* [6]*Department of Neural Circuits and Behavior, Max Delbru¨ck Center for Molecular Medicine, Berlin, Germany*

Abstract. Lymphatic filariasis (LF) is a parasitic infection, caused by three closely related nematodes, namely *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. Previously, we have shown that lysate from *B. malayi* microfilariae induces the expression of interleukin *(IL)-10* and programmed death-ligand *(PD-L) 1* on monocytes, which lead to inhibition of $CD4^+$ T-cell responses. In this study, we investigated associations of *IL-10* and programmed cell death *(PD)-1* pathway gene polymorphisms with clinical manifestation in LF. We evaluated the frequency of alleles and genotypes of *IL-10* (rs3024496, rs1800872), *IL-10RA* (rs3135932), *IL-10RB* (rs2834167), *PD-1* (rs2227982, rs10204525), *PD-L1* (rs4143815), *PD-L2* (rs7854413), and single-nucleotide polymorphisms (SNPs) in 103 patients with chronic pathology (CP), such as elephantiasis or hydrocele and 106 endemic normal (EN) individuals from a South Indian population living in an area endemic for LF. Deviations from the Hardy–Weinberg equilibrium were tested, and we found a significant difference between the frequency of polymorphisms in *PD-L2* (rs7854413; $P < 0.001$) and *IL-10RB* (rs2834167; $P = 0.012$) between the CP and the EN group, whereas there were no significant differences found among *IL-10*, *IL-10RA*, *PD-1*, and *PD-L1* SNPs. A multivariate analysis showed that the existence of a CC genotype in *PD-L2* SNP rs7854413 is associated with a higher risk of developing CP (OR: 2.942; 95% confidence interval [CI]: 0.957–9.046; $P = 0.06$). Altogether, these data indicate that a genetically determined individual difference in a non-synonymous missense SNP of *PD-L2* might influence the susceptibility to CP.

INTRODUCTION

Lymphatic filariasis (LF) is a mosquito-borne, deforming and disabling parasitic disease that is widespread in tropical and subtropical regions, such as sub-Saharan Africa, Southeast Asia, and India. The life cycle of parasitic nematodes that cause LF involves both mosquito vectors and human hosts. Individuals infected with these filarial parasites present a wide spectrum of clinical manifestations ranging from clinically asymptomatic disease associated with microfilaraemia to chronic lymphatic pathology involving lymphoedema, elephantiasis, and hydrocele.[1] Globally, approximately 120 million people are subclinically infected and about 40 million people have chronic LF.[2] Moreover, India accounts for around 40% of the global LF burden (http://apps.who.int/neglected_diseases/ntddata/lf/lf.html). These numbers are static in the literature since decades, despite the strict implementation of mass drug administration (MDA) with anti-filarial drugs to eliminate LF in endemic areas. However, since 2000, only 20 of the 72 endemic countries are now under post-MDA surveillance to demonstrate that elimination has been achieved, and for the remaining countries effective MDA regimen is still in progress.[3] Importantly, a recent publication on the current perspectives of MDA to eliminate LF estimates a 59% fall in the prevalence with close to 100 million cases of LF being prevented or cured after the implementation of MDA.[4]

Chronic infection is characterized by immune dysregulation involving both innate and adaptive immune responses.[5] Asymptomatic infection is associated with a T helper (Th) 2-dependent immune response with a concurrent reduction of Th1 responses followed by a regulated response[6–9]; asymptomatic individuals have impaired monocyte and $CD4^+$ T-cell function as reflected by their inability to produce inflammatory cytokines in response to activating stimuli,[10,11] and elevated levels of interleukin (IL)-10.[12,13] Previously, we demonstrated that monocytes from asymptomatic individuals harboring microfilariae from *Brugia malayi* upregulate programmed death-ligands 1 and 2 (*PD-L1* and *PD-L2*, also known as B7-H1 and B7-DC, respectively), and *IL-10*, which is recapitulated by monocytes from healthy individuals from non-endemic regions when stimulated with *B. malayi* microfilarial lysate in vitro. Importantly, these regulatory markers were capable of inhibiting autologous $CD4^+$ T-cell functions effectively.[13]

The cellular receptor programmed cell death (PD)-1 is an immune-inhibitory receptor expressed by activated T cells, B cells, and myeloid cells. The ligands for *PD-1* (*PD-L1* and *PD-L2*) are type I trans-membrane proteins, structurally related to the B7 family. Programmed death-ligand 1 and *PD-L2* are expressed on a variety of cells; expression on dendritic cells (DCs) and on other cell types down-regulates T-cell immune responses.[14–17] The interaction of *PD-1* with *PD-L1* and *PD-L2* results in inhibition of T-cell receptor–mediated lymphocyte proliferation and cytokine secretion, and blockade of CD28-mediated co-stimulation.[15,18–21] In addition, the anti-inflammatory cytokine *IL-10* plays a major role in regulating inflammatory diseases, such as allergies and autoimmune disorders.[22] Interleukin-10 is secreted by myeloid cells, B cells, and lymphocytes, and suppresses the production of pro-inflammatory cytokines and the activation of Th cells.[23–25] The *IL-10* receptor complex is composed of *IL-10* receptor 1 (*IL-10RA*) and *IL-10* receptor 2 (*IL-10RB*). Interleukin-10 first binds to *IL-10RA* inducing a conformational change that enables *IL-10RB* to interact with the *IL-10*/*IL-10RA* complex.[25] The

* Address correspondence to Svenja Steinfelder, Department of Neural Circuits and Behavior, Max Delbrück Center for Molecular Medicine, NWFZ, Charitéplatz 1, Berlin 10117, Germany. E-mail: svenja.steinfelder@mdc-berlin.de

frequency of T cells expressing *IL-10*[26] and plasma cytokine levels of *IL-10*[27] has been shown to be elevated in asymptomatic cases of LF, as compared with uninfected individuals.[28]

Generally, LF is considered to be a complex, multifactorial disease with a broad spectrum of clinical phenotypes associated with both genetic and environmental factors, which might play a role in determining disease outcome.[29] The identification of potential genetic variants of regulatory molecules involved in mediating host immune responses during various outcomes of LF may inform and improve the development of future therapeutic strategies. A number of genetic studies have provided evidence of associations of *PD-1*–regulated and *IL-10* pathway gene variants with infectious diseases (e.g. extra-pulmonary tuberculosis, influenza, leprosy, and malaria); autoimmune diseases (e.g. ankylosing spondylitis, diabetes mellitus, systemic lupus erythematosus, and ulcerative colitis); as well as asthma and cancer. However, there are currently no known associations of polymorphisms in genes belonging to the *IL-10* and *PD-1* pathway with disease outcome in LF.

With regard to our previous findings, we hypothesized that single-nucleotide polymorphisms (SNPs) in the *PD-1* and *IL-10* pathway genes might be associated with disease susceptibility and/or clinical outcome. Association studies using a candidate-gene approach are the most frequently applied method to identify potential genetic variations modifying risk for disease outcome. In this study, we selected eight SNPs in six candidate genes (*IL-10*, *IL-10RA*, *IL-10RB*, *PD-1*, *PD-L1*, and *PD-L2*) to determine their potential involvement in susceptibility to chronic pathology (CP) in LF and analyzed the frequency of each SNP in a cohort of a South Indian population comprising endemic normal (EN) individuals and patients with CP.

MATERIALS AND METHODS

Study population from India. Samples used for this study were collected in South India, a region that is highly endemic for *Wuchereria bancrofti* infection. Study participants were recruited in cooperation with the National Institutes of Health—International Center for Excellence in Research—National Institute for Research in Tuberculosis (NIRT), Chennai, India, and the Blue Peter Health and Research Center (BPHRC), Hyderabad. We obtained ethical approval for the study from the Institutional Review Board of the NIRT, Chennai (NIRT2013001), and the BPHRC, Hyderabad (project number: 05/2009). Written, informed consent was obtained from all study participants.

DNA extraction. Genomic DNA was obtained from peripheral blood mononuclear cells of patients with CP and EN volunteers using a QIAamp DNA investigator kit (Qiagen, GmBH, Hilden, Germany) or from whole blood using an innuPREP Blood DNA master kit (Analytik Jena AG, Jena, Germany). The quantity and purity of the obtained DNA was confirmed by optical density 260/280 ratios using a Nanodrop spectrophotometer (ND1000; PeQLab Biotechnologie, GmbH, Erlangen, Germany).

Single-nucleotide polymorphism selection and genotyping. We selected two *IL-10* SNPs (rs3024496, rs1800872), one *IL-10RA* SNP (rs3135932), one *IL-10RB* SNP (rs2834167), two *PD-1* SNPs (rs2227982, rs10204525), one *PD-L1* SNP (rs4143815), and one *PD-L2* SNP (rs7854413). We used allele frequency data of a Gujarati Indian population living in Houston, Texas, from the International Haplotype Mapping Project for the selected SNPs in candidate genes (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_viewTable.cgi?pop=12159). The final list of SNPs located within the region of six candidate genes that were selected and analyzed further for a probable association with disease outcome are summarized in the result section. High-purity DNA was used to perform the genotyping analysis by using commercially available hybridization probe–based primers and probes (FastStart master DNA Hybprobe; TIB Molbiol, Berlin, Germany). The genotype results were obtained using a LightCycler 480 II detection system (Roche Diagnostics, Mannheim, Germany). Raw genotype data were uploaded to the statistics database server for association analysis.

Statistical analysis. We analyzed genetic data in patients with CP and EN individuals. The percentage of genotypes in EN individuals was examined for deviation from Hardy–Weinberg equilibrium (HWE) by using a chi-square test. Chi-square test was also used to determine the differences in genotypic and allelic frequencies between patients with CP and EN individuals. Multivariable logistic regression models were used to investigate the influence of SNPs, age, and gender. To choose the best model, co-dominant, dominant, and recessive models were fitted. The model with the lowest 2 log likelihood was selected for further modeling. The OR was calculated to compare the odds of expressing one SNP between the groups. Ninety-five percentage confidence interval [CI] values display the range in which the true OR is located with a probability of 95%. *P*-values smaller than 0.05 were considered statistically significant. The SPSS Statistics 24.0 (SPSS, Inc., Chicago, IL) software was used to analyze the data.

RESULTS

A polymorphism in the *PD-L2* gene is associated with CP in LF. We applied a candidate-gene approach in a south Indian population to evaluate whether SNPs in *IL-10* (rs3024496, rs1800872), *IL-10RA* (rs3135932), *IL-10RB* (rs2834167), *PD-1* (rs2227982, rs10204525), *PD-L1* (rs4143815), and *PD-L2* (rs7854413) genes were associated with susceptibility to LF. Single-nucleotide polymorphisms selected to examine disease association are summarized in Table 1. Inclusion criteria were the following: First, the SNP should be nonsynonymous or present in a putative regulatory region. Second, the allelic frequencies of the selected SNPs should have been registered in the HapMap database for the Gujarati Indian population and have a minor allele frequency of > 0.02.

Demographic characteristics of study population. The study population comprised a total of 209 individuals, including 103 patients with CP and 106 EN individuals. Clinically asymptomatic individuals were excluded from the study because of scarcity of samples (n = 7). Table 2 summarizes the basic demographics of the study population. No significant differences existed at baseline in relation to the gender ratio or mean age values between patients with CP and EN.

Allele frequency analysis. The allelic frequency in all 209 individuals (103 CP patients and 106 EN individuals) was calculated for each of the eight SNPs. Table 3 shows allelic frequencies for the selected SNPs in the CP and EN groups versus those derived from the HapMap database for Gujarati Indians are shown for the selected SNPs. This comparison analysis revealed a higher deviation in allele frequencies (> 0.05%) in the

TABLE 1
Selected SNPs of the candidate genes and their association with diseases

Gene	SNP ID	Type of polymorphism	SNP influence
IL-10	rs3024496	39 UTR	Associated with reduced *IL-10* production in *Ascaris lumbricoides*–stimulated peripheral blood mononuclear cells[30]; associated with *Helicobacter pylori* infection[31]
IL-10	rs1800872	Promoter	Associated with higher susceptibility and greater risk of developing type II diabetes mellitus[32]; correlated with asthma susceptibility[33]; increased risk for colorectal cancer development[34]; decreased risk of breast cancer[35]
IL-10RA	rs3135932	Missense (Ser159Gly)	Associated with extra-pulmonary tuberculosis[36]
IL-10RB	rs2834167	Missense (Lys175Glu)	Associated with systemic sclerosis[37]; systemic lupus erythematosus[38]; benign prostate hyperplasia[39]; ischemic stroke with hypertension[40]
PD-1	rs2227982	Missense (Val215Ala)	Associated with ankylosing spondylitis[41,42]; decreased the risk of breast cancer[43]
PD-1	rs10204525	39 UTR	Increased risk of oesophageal squamous cell carcinoma development[44]
PD-L1	rs4143815	39 UTR	Associated with the risk of gastric adenocarcinoma[45]; type 1 diabetes mellitus in Chilean population[46]; *PD-L1* overexpression in gastric cancer[47]; early stage non-small cell lung cancer after surgical resection[48]
PD-L2	rs7854413	Missense (Ile241Thr)	Not associated with systemic lupus erythematosus[49]

IL = interleukin; PD = programmed cell death; PD-L = programmed death-ligand; rs = reference SNP cluster ID number obtained from dbSNP; SNP = single-nucleotide polymorphism; UTR = untranslated region. The positive or negative association of the genetic variants from regulatory pathway genes with infectious diseases, inflammatory diseases, and cancer conditions are listed.

HapMap population versus our study population for *IL-10* (rs3024496, rs1800872), *IL-10RB* (rs2834167), and *PD-L2* (rs7854413) SNPs, whereas SNPs from *IL-10RA*, *PD-1*, and *PD-L1* did not vary substantially.

Case–control association analysis. Next, we investigated whether the allelic frequencies of the EN group were distributed equally with patients with CP using a HWE and the respective chi-square test. The frequencies of the major alleles, heterozygous and minor alleles, and the *P*-values for eight SNPs, are reported in Table 4. Statistically significant differences were found for the polymorphisms in *IL-10RB* and *PD-L2* between EN individuals and patients with CP ($P = 0.012$ and < 0.001, respectively). Regarding *IL-10RB*, AG and GG genotypes were observed more frequently in the EN group than expected, whereas the AA genotype was observed less frequently. For the *PD-L2* SNP, the genotypes CC and CT in EN individuals occurred more frequently than expected. The Cochran-Armitage test for trends in proportions also confirmed significant differences between the EN and CP groups for the *PD-L2* SNP. We failed to detect any significant differences between the EN and CP groups regarding the genotypes of any other SNPs except rs7854413, which revealed a *P*-value of 0.08 (Table 5).

Multivariable logistic regression models were fitted including SNP genotype as influence factor, and gender and age as confounding factors on the probability to be in group EN compared with group CP. Age significantly increased the probability to be in group CP ($P < 0.001$); however, gender did not (*P*-values between 0.154 and 0.221, Table 6). Although not formally statistically significant, an influence of a SNP (CC compared with either TT or CT) on the group was observed for *PD-L2* ($P = 0.06$). An adjusted odds ratio (aOR) of 2.942 (95% confidence interval: 0.957–9.046) showed that the odds for genotype CC was 2.942 times higher for the CP group than for the EN group (Table 6). These data reveal that this rare variant (CC) of rs7854413, which is a non-synonymous SNP located in the coding region of the *PD-L2* gene, appears to contribute to the risk of developing chronic lymphatic filariasis in a South Indian population.

DISCUSSION

The principle finding of this study demonstrates an association between the rs7854413 polymorphism of the *PD-L2* gene and increased risk of developing pathology in LF in a South Indian population. In the present study, we elucidated a possible association of immune components, which are considered to be vital elements in host immune responses during LF infection. To our knowledge, this is the first case–control study examining an association of SNPs in the *IL-10* and *PD-1* pathway genes with human filarial infections, as many studies have previously reported the association of SNPs from these pathway genes mostly with autoimmune diseases and various cancer conditions, but not with human filarial infections. On the other hand, some studies have reported on genetic variants in other immune-related genes suggesting a strong role in disease outcomes of filarial infection: A transforming growth factor-1 variant was associated with a lack of microfilariae in the blood of asymptomatic individuals,[50] a gene variant in chitotriosidase (*CHIT1*) with susceptibility to human filarial infection and a variant from C-type collectin, mannose-binding lectin (*MBL2*) was associated with protection against filarial infection in a South Indian population.[51] However, another study reported a non-correlation of SNPs in *CHIT1*, toll-like receptor (*TLR*) *2* and *TLR4* with infection status or LF disease phenotype in a Melanesian population.[52] Aside from this, SNPs in the cytotoxic T-lymphocyte antigen-4 promoter gene were associated

TABLE 2
Study population

	Endemic normal ($n = 103$)				Chronic pathology ($n = 103$)			
Cohorts	Male, *n* (%)	Female, *n* (%)	Age, years (mean ± SD)	Age range, years	Male, *n* (%)	Female, *n* (%)	Age, years (mean ± SD)	Age range, years
Hyderabad	22 (65)	12 (35)	40.9 ± 12.61	18–64	11 (32)	23 (68)	49.9 ± 12.01	28–74
Chennai	34 (49)	35 (51)	37.5 ± 11.67	20–64	38 (55)	31 (45)	46.6 ± 12.35	22–70
Total count	56 (54)	47 (46)	38.6 ± 12.09	18–64	49 (48)	54 (52)	47.7 ± 12.28	22–74

SD = standard deviation. Population demographics are shown for the control group of endemic normal individuals and patients with chronic pathology.

TABLE 3
Allele frequencies of selected polymorphisms from patients and control individuals

Gene	SNP ID	Alleles	Ancestral allele	AF (EN)	AF (CP)	AF (HapMap)	AF (EN + CP)
IL-10	rs3024496	C/T	C	C = 0.203	C = 0.209	C = 0.284	C = 0.206
				T = 0.797	T = 0.791	T = 0.716	T = 0.794
IL-10	rs1800872	A/C	C	A = 0.443	A = 0.485	A = 0.381	A = 0.464
				C = 0.557	C = 0.515	C = 0.619	C = 0.536
IL-10RA	rs3135932	A/G	A	A = 0.839	A = 0.855	A = 0.805	A = 0.847
				G = 0.161	G = 0.145	G = 0.195	G = 0.153
IL-10RB	rs2834167	A/G	A	A = 0.665	A = 0.597	A = 0.568	A = 0.631
				G = 0.335	G = 0.403	G = 0.432	G = 0.369
PD-1	rs2227982	C/T	C	C = 0.929	C = 0.927	C = 0.972	C = 0.928
				T = 0.071	T = 0.073	T = 0.028	T = 0.072
PD-1	rs10204525	A/G	A	A = 0.151	A = 0.189	A = 0.182	A = 0.170
				G = 0.849	G = 0.811	G = 0.818	G = 0.830
PD-L1	rs4143815	C/G	G	C = 0.151	C = 0.141	C = 0.148	C = 0.146
				G = 0.849	G = 0.859	G = 0.852	G = 0.854
PD-L2	rs7854413	C/T	T	C = 0.217	C = 0.310	C = 0.244	C = 0.264
				T = 0.783	T = 0.690	T = 0.756	T = 0.737

AF = allele frequency; CP = chronic pathology; EN = endemic normal; rs = reference SNP cluster ID number obtained from dbSNP; SNP = single-nucleotide polymorphism. Comparative analysis of AF between our study population and the HapMap database registered for Gujarati Indians for the selected polymorphisms in the *IL-10* and *PD-1* pathway genes.

with susceptibility to human LF in an east Malaysian population[53] and a positive association was noted between plasma vascular endothelial growth factor-A gene polymorphism and hydrocele development in patients with LF patients from Ghana.[54] In addition, the association of human leukocyte antigen (*HLA*) gene variants with elephantiasis has been demonstrated among an Asian population,[29] and significant differences in the frequency of the *HLAB15* antigen have been shown between patients with elephantiasis and endemic controls in Sri Lanka and South India.[55] Furthermore, an association has been reported between localized onchodermatitis and nsSNPs in the *IL-13* gene.[56]

Our study results reveal that selected SNPs in putative promoter regions of the human *IL-10* gene and variations that cause a missense mutation failed to show any association with LF. Notably, no significant associations with clinical outcomes for LF were found for the SNPs within the *IL-10RA* and *IL-10RB* regions. However, numerous other SNPs within the *IL-10* gene have shown a strong association with many diseases in different study populations. These include: benign prostate hyperplasia (BPH) in a Korean population[39]; leprosy[57]; susceptibility to tuberculosis in Indian human immunodeficiency virus–positive individuals[58]; malaria in young children from Southern Mozambique[59]; asthma susceptibility in an Asian population[33]; and risk of developing hepatitis C virus infection in a Chinese population.[60] An association exists between SNPs in the *IL-10RA* gene and risk of developing extra-pulmonary tuberculosis in Tunisian individuals.[36] There is also an association between *IL-10RA* gene SNPs and the risk of developing cervical adenocarcinoma cancer.[61] Interleukin-10 receptor 2 gene SNPs are associated with autoimmune diseases, such as systemic sclerosis,[37] systemic lupus erythematosus,[38] BPH[39] hypertension, and the risk of ischemic stroke.[39,40] The above-mentioned SNPs within the *IL-10* pathway genes and their possible disease associations largely vary not only between the different conditions, but also depend on the study populations involved.

In agreement with our primary objective, rs7854413, a nonsynonymous SNP within *PD-L2*, was associated with an increased risk of developing pathology in patients with LF compared with EN individuals (Table 6). As mentioned previously there are currently no published data studying the

TABLE 4
Association between SNPs of candidate genes and patients with CP in the study cohort

				Genotypic analysis			
Gene	SNP ID	Group	HWE	pp	pq	qq	*P*-value
IL-10	rs3024496	EN	0.122	68 (0.64)	33 (0.31)	5 (0.05)	0.886
	C/T	CP		67 (0.65)	29 (0.28)	7 (0.07)	
IL-10	rs1800872	EN	0.151	37 (0.35)	44 (0.42)	25 (0.23)	0.415
	A/C	CP		29 (0.28)	48 (0.47)	26 (0.25)	
IL-10RA	rs3135932	EN	0.399	77 (0.73)	24 (0.23)	5 (0.05)	0.692
	A/G	CP		76 (0.74)	24 (0.23)	3 (0.03)	
IL-10RB	rs2834167	EN	0.012	46 (0.43)	49 (0.46)	11 (0.10)	0.131
	A/G	CP		33 (0.32)	57 (0.55)	13 (0.13)	
PD-1	rs2227982	EN	0.488	93 (0.88)	11 (0.10)	2 (0.02)	0.939
	C/T	CP		89 (0.86)	13 (0.13)	1 (0.01)	
PD-1	rs10204525	EN	0.115	76 (0.72)	28 (0.26)	2 (0.02)	0.296
	A/G	CP		68 (0.66)	31 (0.30)	4 (0.04)	
PD-L1	rs4143815	EN	0.117	77 (0.73)	26 (0.24)	3 (0.03)	0.779
	C/G	CP		78 (0.76)	21 (0.20)	4 (0.04)	
PD-L2	rs7854413	EN	< 0.001	65 (0.61)	36 (0.34)	5 (0.05)	0.037
	C/T	CP		52 (0.50)	38 (0.37)	13 (0.13)	

CP = chronic pathology; EN = endemic normal; HWE = Hardy–Weinberg equilibrium; SNP = single-nucleotide polymorphism. Bold values represent statistic significance between the EN individuals and CP patients for IL-10RB and PD-L2 SNPs. Genotype and allelic frequencies for *IL-10* and *PD-1* pathway gene polymorphisms among chronic pathology and control groups were studied using the HWE and association between the SNP and CP was determined.

TABLE 5
Case–control genetic association

Gene	SNP ID	Chi-square statistic (χ^2)	*P*-value
IL-10	rs3024496	0.556	0.757
IL-10	rs1800872	1.120	0.571
IL-10RA	rs3135932	0.464	0.863
IL-10RB	rs2834167	2.867	0.238
PD-1	rs2227982	0.545	[illegible]
PD-1	rs10204525	1.221	0.543
PD-L1	rs4143815	0.638	0.727
PD-L2	rs7854413	5.012	0.082

CP = chronic pathology; EN = endemic normal; SNP = single-nucleotide polymorphism. Bold values represent the significant differences between EN individuals and CP patients for genotypes of PD-L2 SNP. Genotype differences in selected SNPs between EN individuals and patients with CP were tested using the χ^2 test statistic.

association of *PD-L2* gene variant with infectious diseases or in cancers, because most studies focused mainly on the associations of *PD-1* and *PD-L1* gene variants. This might be because of the previously well-demonstrated immune-inhibitory function of *PD-L1* on immune cells in both mice and humans. Besides this, the upregulation of *PD-L2* is mostly restricted to activated antigen-presenting cells including monocytes, macrophages, and DCs.[62] In recent years, the focus has increased considerably toward the understanding of the exact immune mechanisms of *PD-L2* and its influence on other immune cells. Most published results suggest that an engagement of *PD-1* by *PD-L2* dramatically inhibits T-cell receptor–mediated proliferation and cytokine production by $CD4^+$ T cells.[18,63,64] Also, the effect of *PD-L2* in modulating asthma severity by inhibiting allergen-driven IL-12 production in DCs has been described.[65] In addition, the role of *PD-L2*–expressing DCs was demonstrated in a chronic *Schistosoma mansoni* infection model in mice.[66] In parallel, a few studies with conflicting results have been published on the role of *PD-L2* and its specific function on other immune cells. Possible functional differences in the *PD-L2*/*PD-1* versus *PD-L1*/*PD-1* complexes are suggested by studies demonstrating differential upregulation of *PD-L1* and *PD-L2* by Th1/Th2 environments on inflammatory macrophages[67] and detailed insights into the complex interfaces using crystallography.[68] A recently published study on differences in the molecular mechanisms of *PD-L1* and *PD-L2* and their interactions with *PD-1*[69] suggests differential interactions between *PD-L2* and *PD-1* compared with *PD-L1* and *PD-1*. Moreover, a study has previously demonstrated that the interactions of *PD-L1*/*PD-1*, but not *PD-L2*/*PD-1* are essential in diminishing T-cell responses in experimental autoimmune encephalomyelitis (EAE) because cells from *PD-1* and *PD-L1* knockout mice produced higher levels of the pro-inflammatory cytokines interferon-γ, tumor necrosis factor, IL-6, and IL-17, and also developed severe EAE compared with wild-type and *PD-L2* knockout mice.[70] Furthermore, there was a study demonstrating the contribution of *PD-L2* on establishing parasite-specific $CD4^+$ T-cell responses in mice, which protects against lethal malaria. And in the same study, they showed that *PD-L2* expression was important for parasite control as higher frequencies of *PD-L2*–expressing DCs were associated with lower parasitemia in malaria-infected volunteers. This study suggests that *PD-L2* can out-compete *PD-L1* for *PD-1* binding and thus inhibit *PD-L1* functions that were reported to inhibit Th1 cell responses.[71]

In addition, there was a report suggesting an interaction of *PD-L2* with an alternative receptor on T cells—repulsive guidance molecule b (RGMb). Repulsive guidance molecule b induces respiratory tolerance in ovalbumin-exposed mice, as blockade of RGMb-PD-L2 interaction impairs the development of respiratory tolerance significantly.[72,73] Altogether, these results disclose multifaceted consequences of *PD-L2* expression and function, which is distinct from those of *PD-L1*. Finally, the precise mechanism of how *PD-L2* and different polymorphisms affect the function of T cells or possibly other immune cells expressing its receptors in the context of LF remains to be elucidated.

CONCLUSION

Based on our data, a significant association between nsSNP rs7854413 in *PD-L2* and CP with an OR value of 2.9 for the "CC" genotype indicate that a mutation in the *PD-1* pathway genes and its downstream effector mechanisms could play a role in the development of the various clinical manifestations of LF. Our data reveal that individuals differing in this genetic variant (CC genotype) in the *PD-L2* gene are three times more prone to develop pathology than EN individuals. Therefore, we further propose that an individual carrying the "CC" genotype in rs7854413 of *PD-L2* gene is unable to provide the appropriate signal to the *PD-1* receptor on T cells because of *PD-L2* incompetency and/or partial impairment in ligand-receptor interaction. Furthermore, this could eventually lead to a disruption of immune-inhibitory signals via the *PD-1* pathway resulting in active T-cell responses, which engender an inflammatory phenotype within the T cells to challenge the filarial parasite as commonly seen in patients with CP. However, the exact ligand–receptor interactions and the actual immune mechanism of the *PD-L2*/*PD-1*

TABLE 6
Multivariate logistic regression analysis including SNP, age, and gender

				SNP			Age	Gender
Gene	SNP ID	Genotype	Co-dominant analysis	Adjusted odds ratio	95% confidence interval	*P*-value	*P*-value	*P*-value
IL-10	rs3024496	CC	CT or TT	1.970	0.550–7.055	0.297	< 0.001	0.216
IL-10	rs1800872	CC	CA or AA	0.753	0.398–1.422	0.381	< 0.001	0.217
IL-10RA	rs3135932	GG	GA or AA	0.541	0.110–2.661	0.450	< 0.001	0.209
IL-10RB	rs2834167	AA	AG or GG	0.699	0.379–1.289	0.252	< 0.001	0.194
PD-1	rs2227982	TT	CT or CC	0.268	0.023–3.169	0.296	< 0.001	0.173
PD-1	rs10204525	AA	AG or GG	1.813	0.302–10.890	0.515	< 0.001	0.221
PD-L1	rs4143815	CC	CG or GG	0.802	0.398–1.614	0.536	< 0.001	0.220
PD-L2	rs7854413	CC	CT or TT	2.942	0.957–9.046	0.060	< 0.001	0.154

SNP = single-nucleotide polymorphism. Bold values represent the influence of a PD-L2 SNP (CC genotype compared with either TT or CT genotypes). Adjusted odds ratio for factor association with chronic pathology using multivariable analysis.

pathway in terms of disease severity in LF deserves further investigation involving more endemic donors.

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Authors' addresses: Gopinath Venugopal, Noëlle L. O'Regan, and Susanne Hartmann, Centre for Infection Medicine, Institute of Immunology, Freie Universität Berlin, Berlin, Germany, E-mails: gopinath.v@fu-berlin.de, noelleoregan@gmail.com, and susanne.hartmann@fu-berlin.de. Subash Babu, National Institutes of Health, National Institute of Research in Tuberculosis (NIRT), International Center for Excellence in Research, Chennai, India, E-mail: sbabu@mail.nih.gov. Ralf R. Schumann, Institut für Mikrobiologie, Charité–Universitätsmedizin, Berlin, Germany, E-mail: ralf.schumann@charite.de. Aparna Srikantam, Blue Peter Public Health and Research Center, LEPRA Society, Hyderabad, India, E-mail: aparna@leprahealthinaction.in. Roswitha Merle, Department of Veterinary Medicine, Institute of Veterinary Epidemiology and Biometry, Epidemiology Group, Freie Universität Berlin, Berlin, Germany, E-mail: roswitha.merle@fu-berlin.de. Svenja Steinfelder, Department of Neural Circuits and Behavior, Max Delbrück Center for Molecular Medicine, Berlin, Germany, E-mail: svenja.steinfelder@mdc-berlin.de.

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3 Discussion

3.1 Aims and findings

The overall aim of this thesis was (i) to determine how filarial cystatin of the human pathogenic filaria *Brugia malayi* (BmCPI-2) contributes to immune hypo-responsiveness in human monocytes and macrophages elicited by microfilaria, and (ii) to investigate the associations of IL-10 and programmed cell death (PD)-1 pathway gene polymorphisms with clinical manifestation in LF.

The main findings are as followed:

Project 1: Differential immunomodulation in human monocytes versus macrophages by filarial cystatin

1. rBmCPI-2 from *B. malayi* has the ability to induce IL-10, IL-8, IL-6 and PD-L1/L2
2. We demonstrated that the induction of IL-10 and PD-L1/PD-L2 in human monocytes by *B. malayi* Mf lysate is majorly dependent on cystatins
3. We identified the induction of IL-10 by rBmCPI-2 and Mf lysate was differently regulated in human monocytes and macrophages, as we noted that rBmCPI-2-induced-IL-10 production was independent of ERK signalling in monocytes, but dependent on ERK signalling in macrophages.
4. We also identified the induction of IL-10 and IL-8 by rBmCPI-2 and Mf stimulated myeloid cell population is dependent on p38

Project 2: Association of a PD-L2 Gene Polymorphism with Chronic Lymphatic Filariasis in a South Indian Cohort

1. We found significant differences in genotypes only for the polymorphism in PD-L2 gene (rs7854413) between EN individuals and patients with CP
2. Also, we found an association between the rs7854413 polymorphism of the PD-L2 gene and increased risk of developing pathology in LF in a South Indian population.

3.2 Filarial nematodes differentially trigger and exploit various signalling pathways to induce immunomodulation in different myeloid cell subsets. (Venugopal et al. 2017)

Our observation in this study provides evidence that cystatin from *B. malayi* Mf lysate induces immunomodulation on human monocytes and macrophages as it was shown that cystatin depleted microfilarial lysate no longer produces significant amounts of IL-10, which was similar to unstimulated controls (Venugopal et al., 2017). Previous findings from our lab suggest that monocytes from *W. bancrofti* exposed individuals harbouring microfilariae upregulate PD-L1 and also produce cytokines like IL-10, IL-8, and IL-6, which was then recapitulated by stimulation with the lysate of *B. malayi* microfilariae (mf) *in vitro* using monocytes and macrophages from non-endemic healthy donors (O'Regan et al., 2014). This study aimed to investigate whether cystatin from *B. malayi* filarial worms are responsible for the immunomodulation seen on human blood monocytes and macrophages by microfilarial lysate, and was associated with susceptibility to filaria- induced immunomodulation and thus the clinical picture. Principally, the longevity and survival of the parasite within the mammalian host was determined by the capacity of helminth parasite to immunomodulate host immune responses, and this was done primarily by using helminth E/S molecules like cystatin (Maizels and Yazdanbakhsh, 2003; Hewitson et al., 2009; Maizels and McSorley, 2016). As previously mentioned, cystatins are well characterised protease inhibitors of helminths which have immunomodulatory potential. Previously, it was shown by many groups that cystatins modulate host immune responses very effectively. Results from our lab showed that cystatin from *A. viteae* down regulate T cell proliferation and also significantly enhance production of IL-10 in murine macrophages (Hartmann et al., 1997; Klotz et al., 2011). On the other hand, rOv17, a recombinant onchocystatin from the human filarial nematode *O. volvulus*, was found to inhibit the expression of HLA-DR proteins as well as the co-stimulatory molecule CD86 on human monocytes, and also showed the potential to contribute to a state of cellular hypo-responsiveness (Schönemeyer et al., 2001). Hence, understanding the modulatory effects on human monocytes by cystatin from Mf lysate was highly desired due to the fact that circulating microfilariae in asymptomatically infected individuals will readily come into contact with blood monocytes.

In this study, we were interested to analyse whether recombinant BmCPI-2 (rBmCPI-2) - modulated monocytes or macrophages have decreased innate responses and/or alter CD4+ T cell effector functions as described for Mf lysate. Subsequently, to determine the contribution of *B. malayi* cystatin in the immunomodulatory effects of Mf lysate, it was necessary to deplete cystatin from *B. malayi* Mf lysate. Primarily, we detected the presence of filarial cystatin in *B. malayi* Mf lysate and confirmed its ability to induce the cytokine such as IL-10, IL-8 and IL-6,

and also confirmed its potential to upregulate the surface expressions of PD-L1 and PD-L2 on human monocytes and macrophages. In parallel, we determined the ability of rBmCPI-2 to induce a similar regulatory phenotype which was seen in asymptomatic individuals in our previous studies (O'Regan et al., 2014). We then used immunoprecipitation to deplete cystatin from *B. malayi* Mf lysate and we used the cystatin depleted Mf lysate in the culture stimulations to characterise the regulatory phenotype from these myeloid subsets of cells.

Generally, cystatin targets human PBMCs and murine macrophages to induce the production of IL-10 and alter the host immune response (Schnoeller et al., 2008; Klotz et al., 2011; Daniłowicz-Luebert et al., 2013). The results presented in this project also indicate that the induction of IL-10 in both human monocytes and macrophages by *B. malayi* Mf lysate is strictly dependent on cystatins.

In recent years, many studies have been reported discussing the therapeutic potential of cystatin in inflammatory disease due to its capacity to modulate the host immune system by suppressing innate immune cells and also due to its enhanced induction of anti-inflammatory IL-10 and transforming growth factor (TGF-β) (Araujo et al., 2004; Turner et al., 2008; Figueiredo et al., 2010). One study using murine macrophages described the potential of filarial cystatin to suppress the allergic effects of an ovalbumin-induced allergic airway hyper-reactivity and DSS-induced colitis (Schnoeller et al., 2008). Moreover, cystatin from another nematode parasite *Ascaris lumbricoides* showed increased expression of IL-10 and TGF-β along with diminished production of IL-6 and TNFα, which ultimately reduced inflammation in a mouse model of DSS-induced colitis (Coronado et al., 2017). Also, a study demonstrated the effect of filarial cystatin inducing high levels of IL-10 by CD4+ T cells in the airways and lungs of mice, led to the reduction in Respiratory Syncytial Virus (RSV)-induced immunopathology (Schuijs et al., 2016). Correspondingly, our study also indicates the partial involvement in the immune regulation process by cystatin as we could show cystatin-dependent expression of PD-L1 and PD-L2 in human monocytes which possibly lead to PD-L1:PD-1 dependent suppression of CD4+ T cell function which was commonly seen with asymptomatic infected individuals (Freeman et al., 2000; Butte et al., 2007; Wilcox et al., 2009; O'Regan et al., 2014).

Likewise, we also noted significantly increased production of IL-8 in whole Mf stimulated monocytes and macrophages when compared to unstimulated controls, however we found an enhanced level of IL-8 was not dependent on cystatins. The increased levels of IL-8 in the Mf stimulated cells might be due to the presence of other molecules present in Mf that are also able to induce this chemokine (Venugopal et al., 2017). Few studies discussed the ability of filarial products to induce IL-8, which are listed below. Lipoproteins from *Wolbachia,* an

intracellular symbiont of *B. malayi* has the ability to induce IL-8 in a TLR2/TLR6 dependent fashion (Turner et al., 2009). Alternatively, results from other studies demonstrated that infective L3 larvae from *B. malayi* could induce IL-8 in a co-culture of DC and keratinocytes (Boyd et al., 2013), but not in DC (Semnani et al., 2004) or epithelial cells (Weinkopff et al., 2014). Besides this, it was shown that live Mf and E/S products from *B. malayi* female worms induce the expression of IL-8 in human DC (Semnani et al., 2003) and monocytes (Weinkopff et al., 2014). Additionally, we reported the significant elevation of IL-6 levels in Mf stimulated monocytes, but not in macrophages. However, we found cystatin-independent expression of IL-6 in monocytes, but presumably some other molecules from whole Mf lysate were responsible for the elevation of this cytokine. For instance, secretory products from *Wolbachia* could also possibly induce IL-6, as this endosymbiotic bacterium is found in the oocytes, embryos and larval stages and in the hypodermis of male and female filarial nematodes. Previously, it was shown that *Wolbachia* bacteria activate the host immune system (Pfarr and Hoerauf, 2006; Turner et al., 2009) and also induce the production of IL-6, TNFα and IL-1β (Taylor et al., 2001). Initially it was thought that the *Wolbachia* membrane contains lipopolysaccharide (LPS) (Cross et al., 2001; Taylor et al., 2001), and this LPS triggers inflammation via TLR4 leading to secretion of various cytokines, including IL-6 by macrophages and endothelial cells (Taylor et al., 2001; Andre et al., 2002). However, later reports highlighted the role of *Wolbachia* lipoproteins (WoLP) and *Wolbachia* surface protein (WSP) in stimulating innate and adaptive immunity and showed increased production of IL- 6 in a TLR2/6 dependent manner (Brattig et al., 2004; Hise et al., 2007; Turner et al., 2009).

Next, we investigated the immune signalling events which are presumed to be regulated by rBmCPI-2 to induce the production of IL-10 and IL-8 in human monocytes and macrophages. We understand from our previous results that IL-10 production in murine macrophages triggered by cystatin from rodent filarial nematode *A. viteae* is tyrosine kinase sensitive, and depends on activation of both MAPK (Klotz et al., 2011). Moreover, ES-62, another E/S product of *A. viteae* modulates activity of MAP kinases (ERK, p38 and JNK), and thus regulates cytokine production (Goodridge et al., 2003; Harnett et al., 2005). Another study from the same lab reported that ES-62 has the ability to induce tyrosine phosphorylation of glycoproteins in murine macrophages (Helen S. Goodridge et al., 2005). An *in vitro* study using LPS-stimulated mice microglia revealed that filarial cystatin induced expression of IL-10 mRNA was dependent on ERK signalling (Behrendt et al., 2016).

Our results demonstrate the differential induction of IL-10 in human monocytes versus macrophages as we showed that IL-10 induction by rBmCPI-2 and Mf in human monocytes was independent of ERK signalling, whereas it was dependent on both ERK and p38 signalling in monocyte-differentiated macrophages. On contrary, IL-8 production in both monocytes and macrophages was dependent on both MAP kinases (ERK and p38) (Venugopal et al., 2017).

Overall, this data reveals the contribution of filarial cystatin within microfilariae to induce IL-10 in human monocytes, and also shows the ability of filarial products to exploit immune signalling events differentially to induce IL-10 in both monocytes and macrophages. To be precise, cystatin from Mf was sufficient and absolute necessary for IL-10 production, but not so important for IL-8 or IL-6 induction in these myeloid cell populations.

3.3 Investigation on genetic variations of innate immune genes and its associations with clinical manifestations of lymphatic filariasis (LF). (Venugopal et al 2018)

In the present study, we elucidated a possible association of immune components, which are considered to be vital elements in host immune responses during LF infection. Regarding the variances in antigen-specific T-cell responses, spatial variables such as exposure to mosquitoes containing infective larvae and genetic factors play a major role in the outcome of filarial infections. Normally, susceptibility to infection, host response, and risk of development of pathological features cluster in families and are probably driven by host genetics. Therefore, understanding the contribution of host genetics to susceptibility to human lymphatic filariasis led us in the direction of single nucleotide polymorphisms (SNPs) analysis in endemic populations. As mentioned in the previous sections, we have shown that lysate from *B. malayi* microfilaria induces the expression of IL-10 and PD-L1 on human monocytes, which lead to inhibition of CD4+ T cell response (O'Regan et al., 2014). Based on these findings, we selected the following SNPs from candidate genes to investigate the frequency and potential involvement in susceptibility to filarial infections in a cohort of South Indian lymphatic filariasis patients: IL-10 (rs3024496, rs1800872), IL-10RA (rs3135932), IL-10RB (rs2834167), PD-1 (rs2227982, rs10204525), PD-L1(rs4143815) and PD-L2 (rs7854413).

To our knowledge, this is the first case-control study examining an association of SNPs in the IL-10 and PD-1 pathway genes with human filarial infections, as many studies have previously reported the association of SNPs from these two genes mostly with autoimmune diseases and various cancer conditions but not with human filarial infections. We performed genotype analysis using 103 chronically infected LF patients and 106 endemic normal individuals to find possible associations between clinical outcome of LF and SNPs. The key finding of this study revealed a SNP (rs7854413) from PD-L2 gene, which leads to an amino acid exchange, was strongly associated with an increased risk of developing chronic lymphatic pathology (OR = 2.942, 95% CI = 0.957 – 9.046, p = 0.06). Moreover, we identified two SNPs rs7854413 in PD-L2 and rs2834167 in IL-10RB, which are disproportionally distributed between the control and patient groups, whereas no differences were found for the analysed SNPs in IL-10, IL-10RA and PD-L1 genes. So far there were no associations reported with PD-L2 gene SNPs

except one study discussing a SNP in PD-L2 with systemic lupus erythematosus (SC et al., 2007). On the other hand, few studies have reported the IL-10RB gene to be associated with systemic sclerosis (Hikami et al., 2008), systemic lupus erythematosus (Peng et al., 2013), benign prostate hyperplasia (Yoo et al., 2011), and hypertension in the risk of ischemic stroke (Park et al., 2013). Markedly, one study reported that IL-10 has opposite effects on expression of PD-L1 and PD-L2 in AT-2 HIV treated macrophages (Rodriguez-Garcia et al., 2011), and therefore the actual interplay between these two pathways needs to be investigated further in detail.

We did not find any significant association between clinical outcomes of LF and other tested SNPs from IL-10, IL-10RA, PD-1 and PD-L1. But previous studies on various diseases have shown possible association of these SNPs and its direct implication on susceptibility to different clinical conditions was listed below: an association of IL-10 SNP with susceptibility to tuberculosis in an Indian HIV+ individuals (Ramaseri Sunder et al., 2012), malaria in young children from Southern Mozambique (Zhang et al., 2012), susceptibility to asthma in an Asian population (Zheng et al., 2014), benign prostate hyperplasia (BPH) in a Korean population (Yoo et al., 2011), risk in developing hepatitis C virus infection in a Chinese population (Li et al., 2012) and leprosy (Aggarwal et al., 2011) was noted. Also, SNPs from IL-10RA gene showed an association with risk of developing extra pulmonary tuberculosis in Tunisian individuals (Ben-Selma et al., 2012), and risk of developing cervical adenocarcinoma cancer (Hussain et al., 2013), while SNPs from IL-10RB gene were shown to be associated with autoimmune diseases, such as systemic sclerosis (Hikami et al., 2008), systemic lupus erythematosus (Peng et al., 2013), BPH (Yoo et al., 2011), hypertension and risk of ischemic stroke (Yoo et al., 2011; Park et al., 2013).

Based on our findings, the risk of developing pathology in patients with LF when compared to EN individuals was strongly associated rs7854413, a non-synonymous SNP within the *PD-L2* gene. Currently there are no published data available except our report studying the association PD-L2 gene variant, while most studies focused on studying the associations of PD-1 and PD-L1 in infectious disease and in a few cancer conditions since the mechanism of PD-1:PD-L1 and its immune-inhibitory functions on immune cells in both mice and human has been well demonstrated previously. Moreover, PD-L2 upregulation was limited to activated antigen presenting cells which include monocytes, macrophages, and DCs (Yamazaki et al., 2002). But in recent times, the attention towards PD-L2 was gradually increased to better understand its mechanistic role in immune regulation with other immune cells. Previously, it was shown that an engagement of PD-1 by PD-L2 dramatically inhibits T cell receptor mediated proliferation and cytokine production by $CD4^+$ T cells (Latchman et al., 2001; Saunders et al., 2005; Review, 2012). Recently, another study stated the effect PD-L2 mediated inhibition of allergen-driven IL-12 production in DCs to modulate asthma (Lewkowich

et al., 2013). Contradictorily, a few studies reported the differential upregulation of PD-L1 and PD-L2 by Th1/Th2 environments on inflammatory macrophages (Loke and Allison, 2003) and showed differences in the molecular mechanism of PD-L1 and PD-L2 interactions with its receptor PD-1 (Ghiotto et al., 2010). Overall, these reports suggest the dissimilar interactions between PD-L2 and PD-1 compared with PD-L1 and PD-1. Furthermore, one study exhibited the potential of PD-L1/PD-1 in diminishing T cell responses in experimental autoimmune encephalomyelitis (EAE), but not with PD-L2/PD-1 interaction. In this study, they showed cells from PD-1 and PD-L1 knockout mice developed severe EAE and produced elevated levels of pro-inflammatory cytokines IFN-γ, TNF, IL-6 and IL-17, but at the same time wild-type and PD-L2 knockout mice failed to produce the above cytokines (Carter et al., 2007). And recently, a report revealed protection against lethal malaria and establishment of parasite-specific CD4+ T cell responses in mice by PD-L2 (Karunarathne et al., 2016). However, more studies are highly warranted to understand the mechanism of PD-L2/PD-1 pathway mediated control of immune responses in various infection settings.

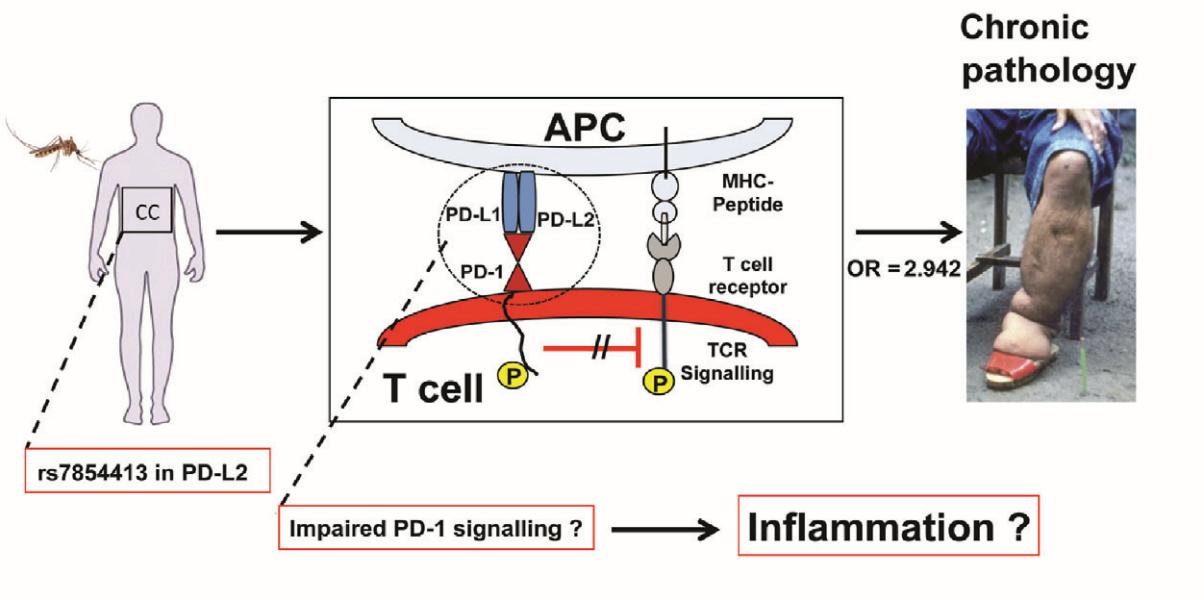
Until thus far, there were only a few published data available on studying the association of a genetic variant in other immune related genes during filarial infections, but not with SNPs from IL-10 and PD-1 pathway genes. Those SNPs and their associations in filarial infections are discussed below: association of a SNP from chitotriosidase (*CHIT1*) with susceptibility to human filarial infection, and a variant from C-type collectin, mannose-binding lectin (*MBL2*) with protection against filarial infection was identified using a south Indian population (Choi et al., 2001). However, another study reported a non-correlation of SNPs in *CHIT1*, TLR2 and TLR4 with infection status or LF disease phenotype in a Melanesian population (Hise et al., 2003). Also, an association of a transforming growth factor- 1 variant with a lack of microfilariae in the blood of asymptomatically infected individuals was reported (Debrah et al., 2011). Additionally, associations of SNPs from cytotoxic T-lymphocyte antigen-4 promoter (CTLA-4) gene with susceptibility to human LF in an east Malaysian population was also found (Idris et al., 2011), and a SNP from plasma vascular endothelial growth factor-A gene (VEGF-A) with hydrocele development in LF patients from Ghana was noted (Debrah et al., 2007). SNP from IL-13 gene was the only cytokine reported an association with filarial infections so far, and it has shown an association with localized onchodermatitis (Hoerauf et al., 2002). Association of SNPs from human leukocyte antigen (*HLA*) gene variants and HLAB15 antigen with elephantiasis in an Asian population were also reported previously (Chan et al., 1984; Choi et al., 2003)

3.3.1 Possible functional implication of rs7854413 SNP in programmed-death ligand 2 gene (PD-L2) in the outcomes of LF – *an outlook*

To understand the exact immunological relationship/mechanism of the PD-L2/PD-1 pathway in terms of disease severity in lymphatic filariasis deserves further investigation and should be functionally examined. The principle finding of this study demonstrated an association between the rs7854413 polymorphism of the PD-L2 gene and risk of developing pathology in lymphatic filariasis in a South Indian population. A significant association between nsSNP rs7854413 in *PD-L2* and chronic pathology with an OR value of 2.9 for the 'CC' genotype indicate that a mutation in the PD-1 pathway genes and its downstream effector mechanisms could play a role in the development of the various clinical manifestations of LF. Based on our data, individuals differing in this genetic variant (CC genotype) in the *PD-L2* gene are three times more prone to develop pathology than EN individuals. Therefore, we further propose that an individual carrying the 'CC' genotype in rs7854413 of *PD-L2* gene is unable to provide the appropriate signal to the PD-1 receptor on T cells due to PD-L2 incompetency and/or partial impairment in ligand-receptor interaction. Furthermore, this could eventually lead to a disruption of immune-inhibitory signals via the PD-1 pathway resulting in active T cell responses, which engender an inflammatory phenotype within the T cells to challenge the filarial parasite as commonly seen in patients with CP (Figure 3). Thus, we aimed to investigate the effect of the 'C' allele in PD-L2 rs7854413 on the immunoinhibitory potential of Mf treated monocytes by performing coculture assay with autologous $CD4^+$ T cells. We needed at least 20 donors who possess this genotype and were willing to provide their blood samples in order to proceed with the functional experiments. Subsequently, we planned to conduct *in vitro* co-culture assays with monocytes from donors harbouring the specific SNP to functionally analyse the PD-L2/PD-1 interaction and its role in dampening immunopathology in filariasis. We would expect a significantly increased production of IFNγ, and IL-17A in CC genotype compared to TT genotype individuals. With regard to that we standardised the functional assay by co-culturing the monocytes with allogeneic CD4+CD45RA-CD25- T cells in the presence of antibodies blocking the PD-L1/L2:PD-1 pathway and we could demonstrate the influence of PD-L2 on the inhibition of alloreactive IL-17+CD4+ T cells. (Figure 4).

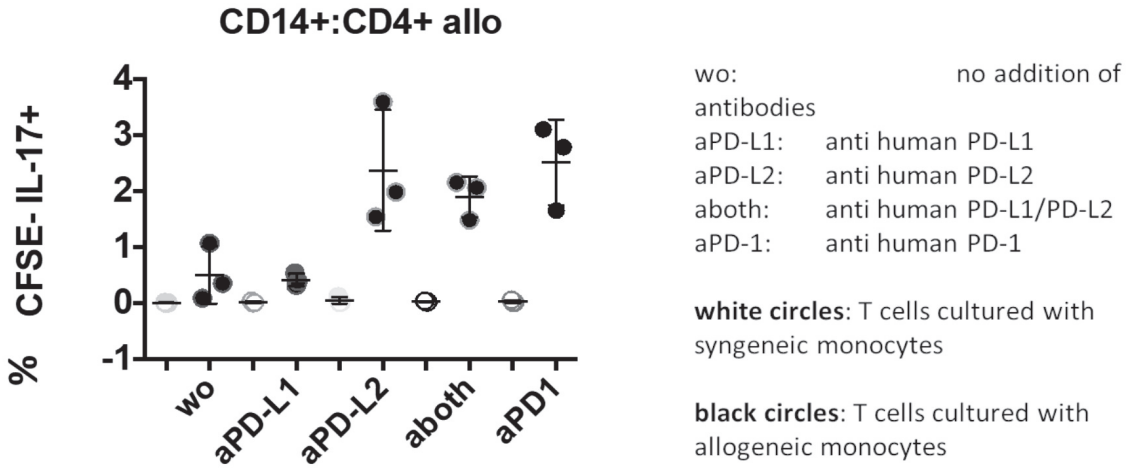
Figure 6. Proposed mechanism based on our results.

The 'CC' genotype in rs7854413 (a nsSNP in PD-L2 gene) might play a crucial role for the uncontrolled inflammation seen with patients with CP. This might be due to the impaired immune-inhibitory signaling of PD-L2 SNP to PD-1 receptor on activated T cells, which could ultimately lead to increased production of pro-inflammatory cytokines.



Abbreviations: APC, antigen presenting cell; CP, chronic pathology patients; MHC, major histocompatibility complex; nsSNP, non-synonymous Single Nucleotide Polymorphism; OR, odds ratio; PD-1, programmed cell death protein; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; TCR, T cell receptor

Figure 7. Co-culture of monocytes with allogeneic CD4+CD45RA-CD25- T cells in the presence of antibodies blocking the PDL1/2: PD1 pathway, n=3



In line of this project, we did genotyping for the SNP that we are interested (rs7854413) in PD-L2 gene with a total of 700 non-endemic healthy volunteers recruited at the Institute of Microbiology and Hygiene of the Charite Berlin, Germany. The genotyped donors served as donors for monocytes and autologous $CD4^+$ T cells to be used in coculture assays. Unfortunately, we could identify only 2% of people bearing this specific genotype among the tested 700 non-endemic individuals, whereas it was about 13% in the endemic population. Moreover, we couldn't reach all those people who possess this genotype for further blood donation to perform our planned functional experiments, thus we limited our study at this point though it deserves further investigation with more endemic donors. However, we strongly believe that *rs7854413* SNP from PD-L2 could play an important role in determining the clinical outcomes of LF and this could possibly be a candidate for a new therapeutic approach in the management of lymphatic filariasis. However, the precise mechanism of how PD-L2 and different polymorphisms affects the function of T-cells and/or other immune cells as well as the actual immune mechanism of the PD-L2/PD-1 pathway in terms of disease severity in lymphatic filariasis remain to be elucidated.

Zusammenfassung

***Brugia malayi* Cystatin-induzierte Immunmodulation an menschlichen Monozyten und Makrophagen und Identifizierung immungenetischer Polymorphismen, die mit lymphatischer Filariose assoziiert sind**

Die Infektionskrankheit Lymphatische Filariose (LF) wird durch *Wuchereria bancrofti, Brugia malayi oder Brugia timori* verursacht und infiziert mehr als 120 Millionen Menschen weltweit. Es wurde gezeigt, dass das Lysat des Larvenstadiums der Mikrofilarien (Mf) humane regulatorische Monozyten und Makrophagen induziert. Mikrofilarien von *Brugia malayi* (mf) führen zu regulatorischen Monozyten mit hochregulierten IL-10 und PD-L1, die in asymptomatisch infizierten Personen auftreten. Frühere Ergebnisse zeigten, dass das Cystatin von Filarien generell die IL-10-Produktion humaner mononukleärer Zellen des peripheren Blutes (PBMCs) und muriner Makrophagen induziert und damit die Immunantwort des Wirtes verändert. *Brugia malayi* cystatin (BmCPI-2) wird in größeren Mengen von *mf* freigesetzt. Deshalb wurde untersucht, wie BmCPI-2 zur Immunhyporesponsivität menschlicher Monozyten und Makrophagen beiträgt, welche durch Mikrofilarien verursacht werden. Daher wurde Cystatin aus dem Mf depletiert und die Wirkung des depletierten Lysats in funktionellen, humanen Zelltests untersucht. Die Analyse des immunmodulatorischen Potenzials von cystatin-depletiertem Mf ergab, dass die Induktion von IL-10, aber nicht von IL-8 und IL-6 in Monozyten und Makrophagen vom Cystatin abhängig ist. Darüber hinaus ist die Mf-induzierte Expression der regulatorischen Oberflächenmarker PD-L1 und PD-L2 in Monozyten, jedoch nicht in Makrophagen von dem Cystatin abhängig. Während Mf-behandelte Monozyten die CD4+ T-Zellproliferation in einem Co-Kultur-Assay verminderten, führte die Stimulation von T-Zellen mit Monozyten, die mit cystatin-depletierten Mf behandelt wurden, zur Wiederherstellung der CD4+ T-Zellproliferation. Darüber hinaus wurde gezeigt, dass die IL-10-Expression in Monozyten im Gegensatz zu Makrophagen, unabhängig vom ERK-Signalweg ist und es daher verschiedene Wege der IL-10-Induktion in diesen Zellpopulationen gibt. Außerdem wird vermutet, dass Genfaktoren eine große Rolle bei der Heterogenität der klinischen Ergebnisse von Filarieninfektionen spielen. Um den Einfluss der Wirtsgenetik auf die Pathogenese von LF zu verstehen, wurden Single Nucleotide Polymorphisms (SNPs) in endemischen Populationen analysiert. Mögliche Assoziationen ausgewählter SNPs in PD-L1, PD-L2, IL-10, IL-10RA und IL-10RB wurden mit dem Krankheitsbild der lymphatischen Filariose in einer südindischen Bevölkerung untersucht. Assoziationstests wurden unter Verwendung logistischer oder linearer Regressionen unter Berücksichtigung des Geschlechts, Alters und pathologischem Status durchgeführt. Es wurden zwei SNPs identifiziert, rs7854413 in PD-L2 und rs2834167 in IL-10RB, welche disproportional zwischen Kontrollgruppe und Patientengruppe verteilt sind. Hingegen wurde für die analysierten SNPs in IL-10, IL-10RA und PD-L1 keine Unterschiede gefunden. Interessanterweise korreliert ein Aminosäurenaustausch im SNP rs7854413 mit einem erhöhten Risiko für die Entwicklung einer chronischen Pathologie (OR = 2.942, 95% CI = 0.957 - 9.046, p = 0.06). Diese Daten zeigen, dass rs7854413 eine Rolle in der Ausprägung der Filarieninfektionen spielen könnte. Weitere funktionelle Studien sind erforderlich, um den beteiligten Immunmechanismus der LF zu verstehen.

Summary

Lymphatic filariasis (LF) is an infection caused by *Wuchereria bancrofti, Brugia malayi or Brugia timori,* a debilitating disease with more than 120 million people infected worldwide. The lysate of the microfilarial larval stage (Mf) was formerly shown to induce human regulatory monocytes and macrophages. *Brugia malayi* microfilaria (mf) induce a regulatory monocyte phenotype with an upregulation of IL-10 and PD-L1 that correlates with asymptomatically infected individuals. Previous results have shown that filarial cystatin generally targets human PBMCs and murine macrophages to induce the production of IL-10 and thereby alter the host immune response. *Brugia malayi* cystatin (BmCPI-2) is known to be released in greater amounts by *Brugia malayi* microfilaria. Thus, here we aimed to determine how *Brugia malayi* cystatin contributes to immune hyporesponsiveness in human monocytes and macrophages elicited by microfilaria. For this purpose, filarial cystatin was depleted from microfilarial lysate and applied in functional cell assays. Detecting the immunomodulatory potential of cystatin-depleted Mf revealed that IL-10, but not IL-8 and IL-6 induction in monocytes and macrophages is dependent on the presence of cystatin. In addition, the Mf-induced expression of the regulatory surface markers PD-L1 and PD-L2 in human monocytes, but not in macrophages, is dependent on cystatin. While Mf-treated monocytes result in decreased $CD4^+$ T-cell proliferation in a co-culture assay, stimulation of T-cells with human monocytes treated with cystatin-depleted Mf leads to a restoration of $CD4^+$ T-cell proliferation. Moreover, we show that IL-10 expression in monocytes, but not in macrophages, is independent of ERK signaling, suggesting different pathways of IL-10 induction in these cell populations. Furthermore, it is assumed that genetic factors play a major role in the heterogeneity of clinical outcomes in filarial infections. Therefore, understanding the contribution of host genetics to the clinical outcome of infection lead in the direction of single nucleotide polymorphisms (SNPs) analysis in endemic populations. We examined the association of selected SNPs in PD-L1, PD-L2, IL-10, IL-10RA, and IL-10RB with disease outcome in patients with lymphatic filariasis in a South Indian population. Association tests were performed by using logistic or linear regression when appropriate, including sex, age and pathological status with the help of SNPStats and SPSS. We have identified two polymorphisms, rs7854413 in PD-L2 and rs2834167 in IL-10RB, which are disproportionally distributed between the control and patient group, whereas no differences were found for the analysed SNPs in IL-10, IL-10RA and PD-L1. Most importantly, the SNP PD-L2 rs7854413 was strongly associated with an increased risk of developing chronic pathology (OR = 2.942, 95% CI = 0.957 – 9.046, p = 0.06). These data indicate that rs7854413 might play a role in the susceptibility to disease outcome in filarial infection and further functional studies are needed to understand the immune mechanism involved.

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Publications & scientific contributions

Publications:

Gopinath Venugopal, Noelle O' Regan, Subash Babu, Ralf R. Schumann, Aparna Srikantam, Roswitha Merle, Susanne Hartmann, and Svenja Steinfelder. ***Association of a PD-L2 gene polymorphism with chronic lymphatic filariasis in a South Indian Cohort.*** Am. J. Trop. Med. Hyg., 100(2), 2019, pp. 344–350

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Immunogenetics of lymphatic filariasis: SNPs in PD-1 and IL-10 pathway genes.
Apr 2016, RoKoDoKo Symposium, Robert Koch Institute, Berlin, Germany

Gopinath Venugopal, Svenja Steinfelder and Susanne Hartmann
Filarial cystatin induced immunomodulation in human monocytes and macrophages.
Mar 2018, German Society for Parasitology (DGP), Berlin, Germany

Gopinath Venugopal, Svenja Steinfelder and Susanne Hartmann
Immunogenetics of lymphatic filariasis: DNA polymorphism in PD-L2 and IL-10R genes. Mar 2016, German Society for Parasitology (DGP), Gottingen, Germany

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Identification of genetic markers linked to susceptibility for filarial-induced immunopathology. WHIP 2015, Woods Hole, MA, USA

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Selbstständigkeitserklärung

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.

Berlin, den 08.08.2019

Gopinath Venugopal