Role of IgG4-mediated Suppression of Immune Effector Mechanisms in Human Filariasis

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Summary

Lymphatic filariasis (LF) is a major public health concern in tropical and subtropical countries. The infection affects more than 120 million people and has significant social and economic consequences on affected individuals and communities. LF is caused by the filarial parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* which are transmitted by mosquito vectors. Filarial parasites are known to be efficient modulators of their host's immune system. To guarantee their own survival, they generate alongside the classical Th2 immune response, a strong regulatory phenotype with high levels of anti-inflammatory cytokines and elevated plasma levels of IgG4. This particular antibody was shown in different models to exhibit immunosuppressive properties and to be associated with the hyporesponsive states observed in LF infection. However, how IgG4 is involved in the pathogenesis of human filariasis is not well characterized. The present thesis aimed at analyzing the role of IgG4 antibody in the suppression of two immune effector mechanisms observed during LF infection: granulocyte activation and degranulation and complement activation. Moreover, the mechanisms sustaining IgG4-mediated immunosuppression were investigated.

The first part of this thesis studied the impact of plasma and affinity-purified IgG/IgG4 fractions from endemic normals (EN), LF infected pathology patients (CP), asymptomatic microfilaraemic (Mf+) and amicrofilaraemic (Mf-) individuals on IgE/IL3/*Bm*Ag activated granulocytes and consequently degranulation. The activation and degranulation states were analyzed by monitoring the expression of CD63/HLADR and the release of granule contents (neutrophil elastase (NE), eosinophil cationic protein (ECP) and histamine) by flow cytometry and ELISA, respectively. The data demonstrated that granulocyte activation and degranulation were inhibited in the presence of plasma from EN and Mf+ individuals, whereas those of Mf- and CP presented no effect. This inhibitory capacity is associated with total IgG and non-IgG fractions of Mf+ patients but was abrogated when non-IgG factors were removed from EN plasma. Strikingly, the inhibitory effect in IgG positive fractions is related to IgG4 antibody. Furthermore, the results also revealed that, except in chronic pathology patients, IgG4 from EN, Mf+ and Mf- selectively reduced the activation of granulocyte neutrophils and basophils but not eosinophils. In the second part, this thesis addressed the question of the mechanisms by which IgG4 suppressed granulocyte functions. IgG4 from Mf+ patients, compared to those from EN and Mf-, demonstrated a high affinity to granulocytes, suggesting possible functional differences between IgG4 antibodies. Moreover, the suppression of granulocyte activation by IgG4 from Mf+ is mediated via FcγRI and

FcγRII and after induction of the phosphorylation of the kinase SHIP1 but not Src and Syk. The third part of the thesis investigated the role of IgG4 antibodies in the modulation of complement activity during LF. The findings indicated that IgG1 and IgG2, present in plasma for Mf+ patients, displayed a reduced capacity to bind complement first component C1q compared to EN, Mf- and CP. Interestingly, the depletion of IgG4 from Mf+ plasma significantly increased the C1q binding capacity of IgG1 and IgG2 suggesting that IgG4 may function by preventing the binding of these pro-inflammatory antibodies to complement.

Taken together these data provide evidences of the participation of IgG4 antibodies in the suppression of granulocyte and complement activities during lymphatic filariasis and also the importance of both qualitative and quantitative modulation of IgG4 in the pathophysiology of LF.

Zusammenfassung

Lymphatische Filariose (LF) ist ein beträchtliches gesundheitliches Problem in tropischen und subtropischen Ländern. Die Infektion betrifft mehr als 120 Millionen Menschen und hat erhebliche soziale und wirtschaftliche Folgen für die betroffenen Personen und Gemeinschaften. LF wird von den Filarien *Wuchereria bancrofti*, *Brugia malayi* und *Brugia timori* verursacht und durch Mückenvektoren übertragen. Filarien sind eine Überfamilie der Fadenwürmer, die dafür bekannt sind, sehr effizient das Immunsystem ihrer Wirte zu modulieren. Um ihr eigenes Überleben zu sichern, induzieren sie neben der klassischen Th2-Immunantwort einen geprägten regulatorischen Phänotyp mit erhöhten anti-inflammatorischen Zytokinen und erhöhtem Plasmaspiegel von IgG4. Dieser spezielle Antikörper wurde in verschiedenen Modellen immunhemmende Eigenschaften zugewiesen, außerdem wurde IgG4 mit den hyporesponsiven Zuständen in LF-Infektion in Verbindung gebracht. Bisher ist jedoch noch nicht genau beschrieben wie IgG4 an der Pathogenese menschlicher Filariose beteiligt ist. Das Ziel der vorliegenden Arbeit war zu untersuchen, ob IgG4 Antikörper durch die Blockierung zweier Immuneffektormechanismen, Granulozytenaktivierung und Degranulation sowie der Komplementaktivierung, eine Rolle im klinischen Spektrum von LF-Infektion spielen. Des Weiteren wurden Mechanismen untersucht, die die immunsuppressive Aktivität des IgG4-Antikörpers unterstützen.

Der erste Teil dieser Arbeit untersuchte die Wirkung von Plasma und aufgereinigten IgG/IgG4-Fraktionen auf IgE/IL-3/*Bm*Ag aktivierte Granulozyten und folglicher Degranulation. Dazu wurden Proben von endemisch Normalen (EN), LF infizierten pathologischen Patienten (CP), asymptomatischen Mikrofilarien-positiven (Mf+) und Mikrofilarien-negativen (Mf-) Personen verwendet. Die Aktivierung und Degranulation wurde anhand der Expression von CD63/HLADR und der Freisetzung des Inhalts von Granulaten (neutrophile Elastase (NE), eosinophilen kationischen Protein (ECP) und Histamin) untersucht und mit Hilfe von Durchflusszytometrie und ELISA analysiert. Die Daten zeigten, dass die Granulozytenaktivierung und Degranulation in Gegenwart von Plasma von EN und Mf+ Individuen gehemmt wurden, während bei den Mf- und CP Patienten keine Inhibition zu sehen war. Diese hemmende Kapazität wurde mit Gesamt-IgG-Fraktionen und mit Fraktionen ohne IgG von Mf+ Patienten in Verbindung gebracht, jedoch war diese Wirkung aufgehoben, wenn nicht-IgG-Faktoren aus dem Plasma der EN Patienten depletiert wurden. Bemerkenswerterweise hängte die hemmende Wirkung der IgG-positiven Fraktionen mit den IgG4-Antikörpern zusammen. Außerdem wiesen die Ergebnisse darauf hin, dass IgG4

von EN, Mf+ und Mf- Individuen, außer CP, die Aktivierung von Granulozyten-Neutrophilen und Basophilen, aber nicht die von Eosinophilen, selektiv reduzierten. Der zweite Teil dieser Doktorarbeit befasste sich mit der Frage nach den Mechanismen, durch die die IgG4-Antikörper die Funktionen von Granulozyten unterdrückten. IgG4 von Mf+ Patienten zeigte im Vergleich zu den anderen untersuchten Gruppen eine hohe Bindungsaffinität gegenüber Granulozyten, was auf mögliche funktionelle Unterschiede zwischen IgG4-Antikörper hindeutet. Darüber hinaus wurde die Unterdrückung der Granulozytenaktivierung durch IgG4 von Mf+ über FcγRI und FcγRII vermittelt und trat ebenfalls nach Induktion der Phosphorylierung der Kinase SHIP1 auf, aber nicht bei Src und Syk. Der dritte Teil dieser Doktorarbeit untersuchte die Rolle der IgG4-Antikörper bei der Modulation der Komplement-Aktivität während LF Infektionen. Die Ergebnisse zeigten, dass IgG1 und IgG2 im Plasma von Mf+ Patienten im Vergleich zu EN, Mf- und CP Individuen, eine reduzierte Bindungskapazität gegenüber der ersten Komponente des Komplements, C1q, aufzeigten. Interessanterweise ist nach der Depletion von IgG4 in Mf+ Plasma die C1q-Bindungskapazität von IgG1 und IgG2 signifikant gestiegen, was darauf hindeutet, dass IgG4 die Bindung dieser pro-inflammatorischen Antikörper zum Komplement verhindern könnte.

Zusammenfassend zeigen die Daten dieser Arbeit, dass IgG4-Antikörper an der Unterdrückung von Granulozyten und der Komplement-Aktivierung während der LF-Infektion beteiligt sind und ebenso die Bedeutung der qualitativen sowie quantitativen Modulation der IgG4-Antikörper in der Pathophysiologie lymphatischer Filariose.

THIS THESIS IS BASED ON THE FOLLOWING ORIGINAL PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

PUBLICATIONS

*Pathological manifestations in lymphatic filariasis correlate with lack of inhibitory properties of IgG4 molecules on IgE-armed granulocytes. **Prodjinotho, U.F.**, von Horn, C., Hoerauf, A., Adjobimey, T. International Congress of Immunology (ICI) 2016. Melbourne, Australia. August 21-26, 2016. Eur. J. Immunol. 2016. 46, S1, p252. Abstract 2875.

***Ulrich F. Prodjinotho** *et al*. Pathological Manifestations in Lymphatic Filariasis Correlate with Lack of Inhibitory Properties of IgG4 Antibodies on IgE-activated Granulocytes. *Manuscript in preparation.*

***Ulrich F. Prodjinotho** *et al*. IgG4 from Filariasis asymptomatic microfilaraemic patients impairs the activation of complement by blocking the binding of IgG1 and IgG2 on C1q. *Manuscript in preparation.*

*IgG4 subclass antibodies produced during lymphatic filariasis impair the activation of IgE-armed neutrophils and basophils but not eosinophils through interaction with FcγRI and II. **Prodjinotho, U.F.**, von Horn, C., Hoerauf, A., Adjobimey, T. 68th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM 2016). Ulm, Germany. September 11-14, 2016. IJMM 2016. Abstract 101/IIV. *Abstracts to be published in International Journal of Medical Microbiology.*

SCIENTIFIC CONTRIBUTIONS

Oral presentations:

**Lack of inhibitory properties of IgG4 molecules on activated granulocytes correlates with pathological manifestations in lymphatic filariasis.* West Africa Regional School on Immunology of Infectious Diseases (ImmunoGambia 2016), 19-26.11.2016, Banjul, The Gambia.

**IgG4 subclass antibodies produced during lymphatic filariasis impair the activation of IgE-armed neutrophils and basophils but not eosinophils through interaction with FcγRI and II*. 68th Annual Meeting of the German Society for Hygiene and Microbiology (DGHM), 11-14.09.2016, Ulm, Germany.

**Pathological manifestations in lymphatic filariasis correlate with lack of inhibitory properties of IgG4 molecules on IgE-armed granulocytes*. 16th International Congress of Immunology (ICI), 21-26.08.2016, Melbourne, Australia.

**Pathological manifestations in lymphatic filariasis correlate with lack of inhibitory properties of IgG4 molecules on IgE-activated granulocytes*. 27th Annual Meeting of the German Society for Parasitology (DGP), 09-12.03.2016, Göttingen, Germany.

Poster presentations:

**IgG4 subclass antibodies produced during lymphatic filariasis impair the activation of IgE-armed neutrophils and basophils but not eosinophils through interaction with FcγRI and II*. 46th Annual Meeting of the German Society for Immunology (DGfI), 27-30.09.2016, Hamburg, Germany.

**Pathological manifestations in lymphatic filariasis correlate with lack of inhibitory properties of IgG4 molecules on IgE-armed granulocytes*. Immunosensation Cluster Science Days, 2-3.11.2015, Bonn, Germany.

**Pathological manifestations in lymphatic filariasis correlate with lack of inhibitory properties of IgG4 antibodies on granulocytes*. 7th DGfI Autumn School of Immunology, 04-09.10.2015, Merseburg, Germany.

List of abbreviations

AAM	Alternatively activated macrophages
ADCC	Antibody-dependent cellular cytotoxicity
AP	Alternative pathway
APC	Allophycocyanin
APS	Ammonium persulfate
BmAg	*Brugia malayi* antigen
Breg	Regulatory B cells
BSA	Bovine Serum Albumin
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CP	Chronic pathology
CP	Classical pathway
CTLA	Cytotoxic T ymphocyte antigen
DAMPs	Damage-associated molecular pattern molecules
ELISA	Enzyme-linked immunosorbent assay
EN	Endemic normal
EU	Endotoxin units
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

Foxp3	Forkhead box protein 3
FSC	Forward scatter
GATA3	GATA binding protein 3
IFN-γ	Interferon gamma
IgG	Immunoglobulin gamma
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
L3	Filarial larvae stage 3
LP	Lectin pathway
LPS	Lipopolysaccharide
mAb	Monoclonal antibodies
MAPK	Mitogen-Activated Protein Kinase
Mf	Microfilariae
MHC	Major Histocompatibility Complex
mL	Milliliter
µL	Microliter
min	Minutes
mM	Millimole
NK	Natural Killer cells
PAMP	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cyanine 5
PFA	Paraformaldehyde
RBC	Red blood cell
Rpm	Rotation per minute
RPMI1640 Medium	Roswell Park Memorial Institute Medium
RT	Room temperature
SDS-page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SSC	Sideward scatter
TBS	Tris-buffered saline
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper cells
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Tregs	Regulatory T cells

Table of Contents

1. INTRODUCTION

Filarial worms are thread-like nematode parasites that are transmitted by insects that feed on blood. The most important filarial diseases are lymphatic filariasis and onchocerciasis, which are major causes of disability in the tropics. The present thesis focuses on lymphatic filariasis and analyses the role of IgG4 antibodies in the inhibition of immune effector mechanisms during lymphatic filariasis.

1.1. Lymphatic filariasis

1.1.1. Cause and geographical distribution

Lymphatic filariasis (LF) also known as elephantiasis is a disabling and profoundly disfiguring infection caused in human by vector-borne nematodes. In 2000, when the Global Programme to Eliminate Lymphatic Filariasis (GPELF) was launched, an estimated 120 million people in 83 countries were infected, of which 40 million suffered from overt diseases [1,2]. These numbers were probably underestimates, as later surveys found that infection rates were much higher than expected in many areas [3]. 90% of these infections are caused by the species *Wuchereria bancrofti*, while the remainder is caused by *Brugia malayi* and to a lesser extent, *Brugia timori*. The disease is prevalent in the tropics and subtropics: sub-Saharan Africa, Southern and South-east Asia, parts of South America, the Caribbean and the South Pacific (Figure 1) and has significant social and economic consequences for affected individuals as well as for their families and communities [4]. While mortality is rare, morbidity is extremely high and results in permanent and long-term damage, characterised by the destruction of the lymphatic vessels, where the adult worms reside [5]. This chronic affection is associated with impaired mobility and social activity, reduced work capacity, sexual dysfunction, severe psycho-social problems, stigma and bad marital prospects [6-8].

Mass drug administration is recommended to interrupt LF transmission and control morbidity for those already afflicted and thereby break the life cycle of filarial worms.

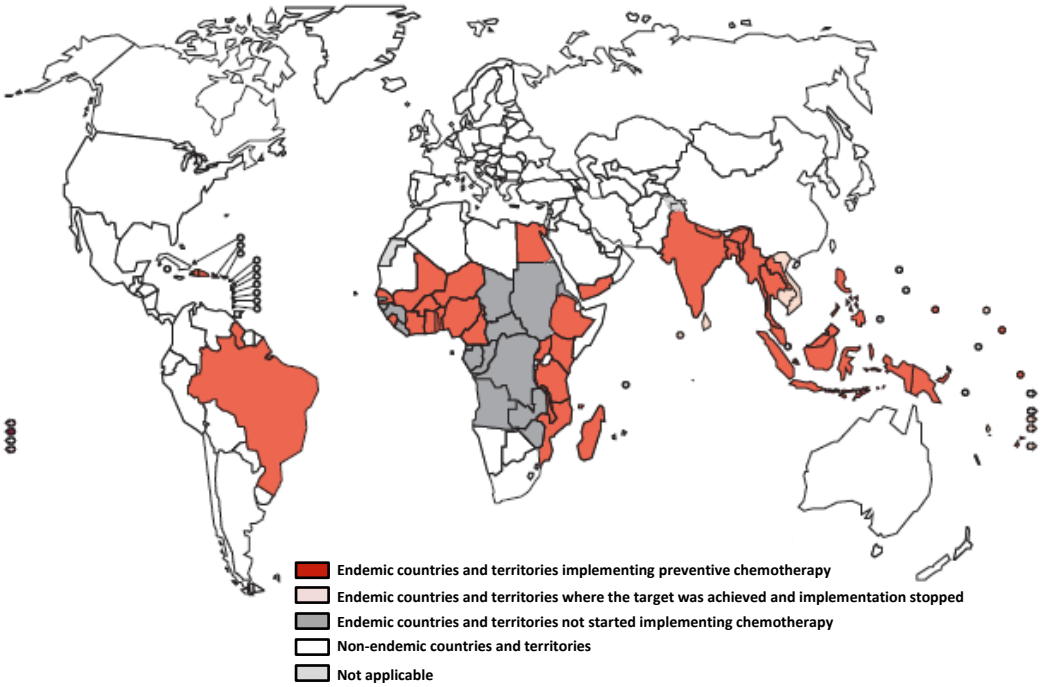

Figure 1: Distribution and status of mass drug administration for lymphatic filariasis worldwide, 2011. Map shows endemic and non-endemic countries of lymphatic filariasis and the status of mass drug administration in these countries during 2011. Obtained from [9]

1.1.2. Life cycle and transmission

The life cycles of *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* are very similar. Humans are the primary reservoirs for lymphatic filariasis. Only *Brugia malayi* has known animal (feline and primate) reservoirs. An example of the life cycle for lymphatic filariasis is shown in Figure 2.

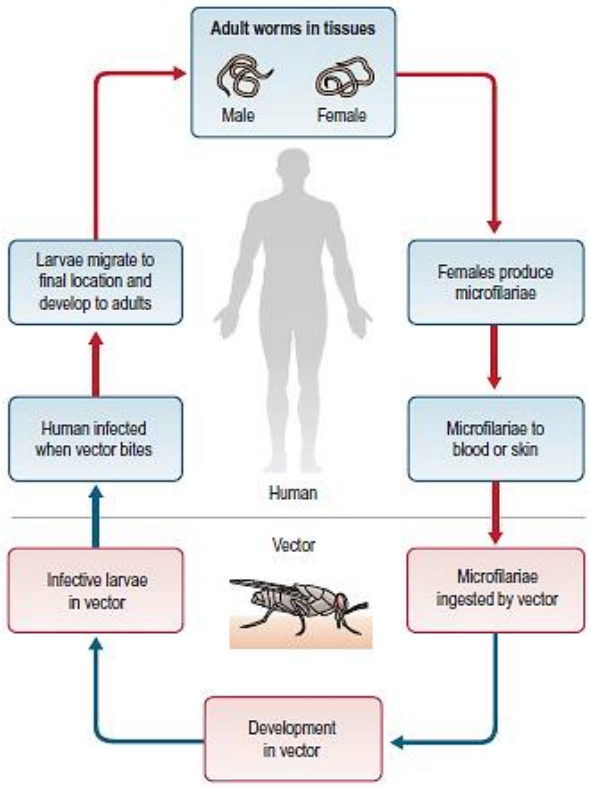

Figure 2: Schematic representation of the life cycle of lymphatic filariasis. The figure shows the development of the parasite in human host and mosquito vector. Obtained from [10].

The adult worms (macrofilaria) reside in the lymphatic system of the human host, where they live for more than 20 years but their average lifespan is shorter. During their lifespan, after mating, females release, in lymphatic vessels, thousands of microfilariae (Mf) that eventually enter the peripheral blood stream [11], from where they are ingested by female mosquito vectors during blood meals. Within the vector the Mf undergo two obligatory molts over a minimum of 12 - 14 days that vary with ambient temperature, to become mature infective third-stage larvae (L3). The infective larvae migrate to the mouthparts of the mosquito from where they are injected into the skin of the human host during a blood meal. The larvae migrate from the skin to the lymphatic system and develop into mature male and female worms. Typically, this occurs in the afferent lymphatics over a period of months, during which time a person may be infected but amicrofilaraemic with no detectable circulating

filarial antigen (CFA) [12,13]. Mf appear in the blood after a minimum of 8 months in *W. bancrofti* and 3 months in *B. malayi* and have a lifespan of approximately 1 year [10]. Mf circulate between the lymphatic vessels and the peripheral blood stream, where they can be taken up again by the transmitting vector.

A mosquito vector transmits the disease and the microfilarial lifecycle stage displays periodicity that is dependent on the blood-feeding patterns of the vector species present in the relevant geographical location [14]. *W. bancrofti* is carried principally by *Culex quinquefasciatus* and by *Aedes* spp.[15]. The principal mosquito vectors for *B. malayi* include *Mansonia* and *Anopheles* mosquitoes. *Anopheles barbirostris* is the only known mosquito vector for *B. timori*[16]. Depending on mosquito vectors and regions, transmission can occur during the day, at night, indoors or outdoors [16]. Microfilariae display predominantly nocturnal periodicity such that they are detected in the bloodstream only during the approximate hours of 21:00 to 04:00. An exception is the Pacific Islands where microfilariae are found in the bloodstream continuously but in varying density depending on the time of day [17,18]. When microfilariae are not in the blood, they are found in deep tissues, particularly the lungs [19,20]. In endemic communities, infection and transmission of LF rates may vary in different sectors and even from one household to the next. Transmission of LF is not only the product of prevalence and intensity of infection in the population but also the vector capacity of the mosquito [21]. Typically, prevalence and intensity are correlated since when there is a low intensity there is less transmission and prevalence declines. However in certain countries, the transmission may persist at low parasite levels [22]. This has the potential to impact on LF elimination in these countries. In most endemic areas the hot months of the rainy season and sometimes summer were found to be the high time for filarial transmission. In addition the development and transmission of the disease, in some areas, may also be favored by water and sanitation [23].

1.1.3. Clinical presentation

In LF infection, the parasite-host relationship is complex, involving a balance between the density of incoming infective larvae, the immunity of the individual and other environmental factors such as vector distribution. Lymphatic dysfunction occurs in filarial infected symptomatic or asymptomatic individuals either due to inflammation and/or secreted/excreted parasite products or mechanical obstruction of the flow of lymph [24,25]. In endemic regions, exposition to the infection leads to different clinical phenotypes. The first clinical group includes putatively immune individuals referred to as "endemic normals" (EN), who remain infection and disease-free despite continuous exposition to mosquito-transmitted infective larvae (L3). These individuals display a robust immune system destroying incoming infective larvae and show evidence of exposure by testing positive by anti-filarial antibody assays [26]. Endemic normal individuals can remain for many years or lifelong infection-free. However it has been demonstrated that 21% of EN individuals could develop infection within a year [27].Those infected individuals, if still asymptomatic, become part of the second clinical group termed "asymptomatic infected individuals".

The group of asymptomatic infected individuals is characterized by the presence of adult worms with no symptoms or signs of disease [28]. It includes asymptomatic individuals with latent infection, who are free of microfilariae (Mf-) and asymptomatic infected individuals who develop microfilareamia (Mf+) with hyporesponsive immune profile but present few visible clinical manifestations despite large numbers of circulating microfilariae [29-31]. People with asymptomatic infections are relatively tolerant to filarial worms and most of them remain infected without clinical symptoms. However, many individuals of this group have subclinical pathology such as lymphangiectasia (dilatation of lymphatic vessels) [32,33].

The third group represents infected individuals presenting clinical manifestations of the disease. There are characterized by the presence of low numbers of parasites and even absence of parasites at later stages of infection, but hyper-reactive immune phenotype that promotes chronic lymphatic pathologies (CP) due to dying parasites [5,34]. In bancroftian filariasis, the most common clinical manifestations are acute adenolymphangitis (ADL) and hydrocoele, lymphoedema and elephantiasis [10] (Figure 3). Chyluria and tropical pulmonary

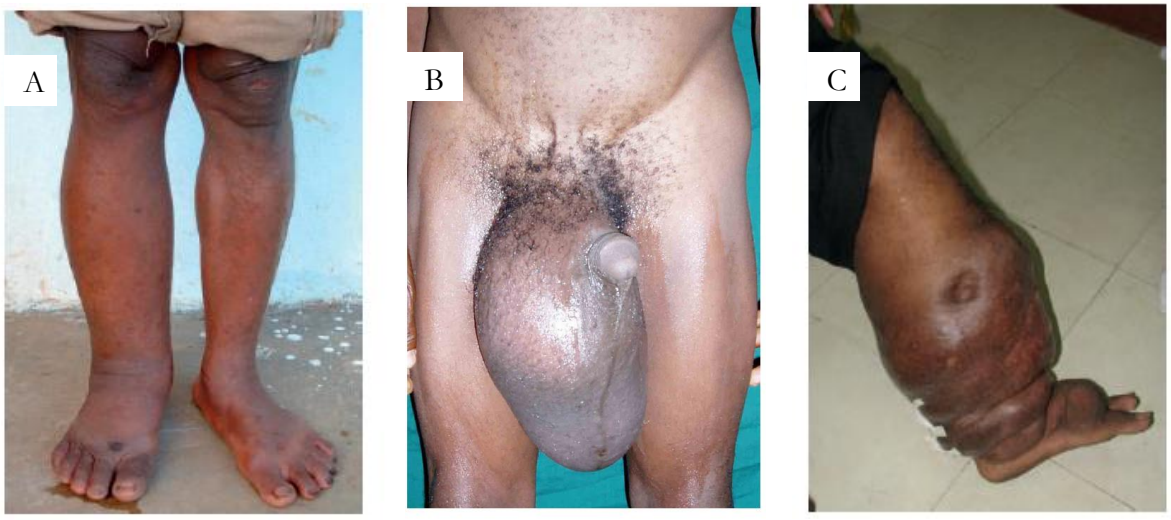

Figure 3: Pathological manifestations of lymphatic filariasis in endemic regions. Examples of disease manifestations are shown. (A) Lymphedema of the right leg (*Brugia Timori*) [10]. (B) Hydrocele (*Wuchereria bancrofti*) [35]. (C) Advanced stage Elephantiasis of left leg (*Wuchereria bancrofti*) [10].

eosinophilia (TPE) are less common [10,28]. Acute manifestations of filariasis was originally thought to only manifest as ADL, but it was discovered that there were two distinct clinical manifestations divided into acute filarial lymphangitis (AFL) and acute dermatolymphangioadenitis ADLA) [36]. Lymphangiectasia caused by adult worms impairs lymphatic function and predisposes the host to microbial and/or secondary bacterial infections that may cause ADLA. ADLA attacks are episodic events that start with malaise, fever and

chills and lead to warm and swollen affected parts. ADLA events usually resolve spontaneously after about a week, but they often recur several times per year [37]. In contrast to ADLA, AFL is believed to be triggered by parasite death, which may occur spontaneously or after treatment and can be either symptomatic or asymptomatic [36]. AFL episodes are typically less severe than ADLA and they rarely lead to long-term lymphoedema. AFL attacks in intrascrotal lymphatic vessels can cause acute transient painful hydroceles with temporary impairment of the lymphatic flow from the tunica vaginalis [38] whereas repeated episodes of ADLA can lead to chronic lymphoedema and elephantiasis. The gradual process leading to chronicity often takes many years [39]. In infected men, hydrocele is the most common chronic clinical abnormality. It results from the accumulation of serous fluid in the tunica vaginalis surrounding the testicles and can be graded according to the developmental stage and size [40] while lymphoedema is the accumulation of lymph due to lymphatic obstruction either due the worm or inflammation. It commonly affects the lower legs. However, the arms, scrotum, penis, vulva and breasts can also be affected. Further progression of chronic lymphedema leads to elephantiasis. Debilitating elephantiasis is often complicated by secondary bacterial and fungal infections, the humid folds of the skin creating a niche for these organisms [41]. The main clinical difference between brugian and bancroftian filariasis is the absence of hydroceles and other genital lesions and chyluria in areas endemic for *B. malayi* and *B. timori* [16].An early diagnosis of these clinical manifestations may help to control the disease. .

1.1.4. Diagnosis of LF

Diagnosis of LF is achieved through a combination of epidemiological history, clinical findings, and laboratory tests. The three main markers for LF diagnosis are microfilaraemia, antigenaemia and/or presence of anti-filarial antibodies. These are completed by several methods including detection of adult worms by ultrasonography and filarial parasites in

mosquitoes. Diagnosis based on Mf detection provides evidence for filarial infection and microfilarial size and morphology can be used to differentiate between different filarial species. Mf assays currently available include thick blood smear, Knott's concentration method, membrane filtration techniques, and, more recently, PCR techniques. But this diagnosis is limited as many patients are amicrofilaraemic and there is no relationship between microfilaria counts in blood and disease severity. Circulating filarial antigen based diagnosis is more sensitive and easy than tests that detect microfilariae [27,42,43]. CFA tests have been developed for diagnosis of *W. bancrofti* infections but are not yet available for Brugian filariasis. These tests detect antigens released by adult *W. bancrofti* worms in human blood, serum, or plasma samples for infected amicrofilaraemic and asymptomatic infected individuals. Commercially available antigen tests include a rapid format card test and an ELISA. In addition, antigen levels remain stable during the day and night, so these tests can be performed at any time. Anti-filarial antibody assays detect elevated levels of IgG and IgG4 but do not differentiate between the various types of filarial infections and often cross-react with antigens from other helminths. Furthermore, these antibody tests cannot distinguish between active infection and past infection or exposure, although several assays based on recombinant antigens appear to have enhanced specificity [44]. Detection of antibodies as diagnostic tool is beneficial for individuals residing in non-endemic areas, since a positive test would be indicative of exposure and the need for treatment.

1.1.5. Treatment and control

Treatments based on mass drug administration (MDA) are part of the strategies recommended by the World Health Organization (WHO) to prevent and control LF. In addition WHO recommends intensified case-management, vector control, provision of safe water, sanitation and hygiene and veterinary public health [23]. Mass drug administration of albendazole together with either ivermectin (IVM) or diethylcarbamazine (DEC) is the main intervention

for controlling morbidity in population at risk of infection. A single dose of albendazole (400 mg) with either DEC (6 mg/kg) or ivermectin (200 μg/kg) significantly reduces microfilaria load. Albendazole with DEC is believed to have better macrofilaricidal activity than albendazole with ivermectin. However, DEC is contraindicated in patients with onchocerciasis; therefore albendazole/ivermectin regimen is preferred for treatment of LF in areas that are co-endemic for onchocerciasis. These strategies have been impressively successful in reducing Mf burden and elimination of lymphatic filarasis in several areas [45]. In addition, doxycycline has been introduced for individual drug administration directed against the *Wolbachia* bacteria of the filariae [46-48]. The antibiotic inhibits filarial embryogenesis and has been proven to be macrofilaricidal and to stop or reduce pathology [46,49,50]. However emerging resistance to IVM [51] reinforces the urgent need for alternative ways of LF control. Vector control with insecticide-treated bed nets is also a valuable tool for *W. bancrofti* elimination in areas where anopheline mosquitoes transmit the parasite along with other personal protection measures. Thus a better understanding of vector biology as well as the host immunity to LF will contribute to developing appropriate treatment for the prevention and control of LF.

1.2. Immunity in human filariasis

1.2.1. Host immune response to LF

After filarial parasite invasion; immune recognition, effectiveness of immune reactivity and protective response are the mechanisms that affect parasite abundance and survival in the host. To boost host protection and based on the early success of vaccines against viral and bacterial infections, several studies using different approaches have been performed to generate an active vaccine against filarial parasites. Although these attempts to immunize animals showed relatively interesting protection, none of these studies reported complete

immune protection and clearance of adult worms or Mf. The immune response to filarial parasites is a complex process involving a delicate balance between a predominant T helper 2 (Th2) response, and in some cases exaggerated T helper 17 (Th17) response, with T helper 1 (Th1) assistance and parasite-induced down-regulation. Host protection against invading filarial worms is defined by a strong Th2-type immune response that destroys and/or expels the parasite. Cells of the innate and adaptive immune system are important for initiation of Th2-type immunity. Th2-type immunity involves a cellular mobilization with an appropriate humoral immunity that includes secreted and excreted proteins such as cytokines, antibodies and the proteins of the complement cascade (section 1.5) [52]. Complement proteins function to directly lyse or opsonize filariae. Opsonisation facilitates parasite recognition by innate immune cells. Certain innate immune cells secrete soluble mediators and cytotoxic granules to lyse filariae whereas others, such as macrophages, serve as antigen presenting cells (APCs) and recruit adaptive immune mechanisms if the innate immune response is unsuccessful in destroying the parasite [52]. The key players in Th2-type immunity are CD4+ Th2 cells and involve the cytokines IL-4, IL-5, IL-9, IL-10, and IL-13; the antibody isotypes IgG1, IgG4 and IgE (section 1.4) and expanded populations of granulocytes (section 1.3) and alternatively activated macrophages [53,54]. Nevertheless, in endemic regions, these responses vary from one group of exposed individuals to another and the breakdown in the delicate balance contributes to the different clinical manifestations of the disease.

The group of chronic lymphatic pathologies (CP) patients is defined by a hyper-reactive phenotype. The patients elicit a strong Th1 and Th17 pro-inflammatory responses that eliminate the microfilarial stage [55] while inducing the production of angiogenic factors like VEGF known to be associated with development of filarial lymphedema [56]. This severe clinical profile is characterized by high antigen-specific immunoglobulin E (IgE) and low IgG4 [55,57,58]. Parasite death and subsequent release of endosymbiont *Wolbachia* products

promote a classical Th1 type response with IFN-γ, IL-6, and TNF-α [59]. This leads to an influx of inflammatory cells in tissue surrounding degraded worms within the lymphatics and causes destruction of lymphatic vessels [28]. In *W. bancrofti*, and *B. malayi* infections, this can result in the development of lymphedema and ultimately elephantiasis or hydrocoele, whereby the lymphatic tissue becomes dilated and hypertrophic. Patients with TPE have exaggerated immune responses directed against microfilariae and filarial antigens in the lung. They display very high serum levels of filaria-specific IgG and IgE antibodies and marked peripheral blood eosinophilia.

In contrast to the hyper-reactive phenotype displayed by chronic lymphatic pathology patients, the group of asymptomatic individuals (Mf+) is associated with a hypo-responsive immune profile. Subjects from this group commonly present alongside the classical Th2 immune response, a strong parasite-specific immunoregulatory phenotype (termed "modified Th2 immune response") allowing the presence of adult worms and/or not microfilariae. This response is defined, in humans, by the development of specific antibody isotypes including, mainly, induction of IgG4 accompanied by a decrease in IgE, IL-4 and IL-5, while IL-10 levels from different regulatory cell sources increase [53]. This is associated with increased number of regulatory T cells (Tregs) and alternatively activated macrophages (AAM) as well as a suppression of Th1 inflammatory cytokines (such as gamma interferon [IFN-γ]) accompanied with the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β that protects the host from immunopathology and permits parasite establishment. This predominantly immunosuppressed environment is associated with elevated levels of antigen specific IgG4 but limited IgE production and is directly linked with filarial parasite survival [60-62]. The outcome of this immune-compromise may not only enhance parasite survival and further infections but may also be beneficial to the host, through limiting immunopathology and reducing allergies and autoimmune diseases [62-65]

Endemic normal individuals (EN) develop a different but mixed response from the two immune phenotypes described above. These individuals typically have a mixed Th1/Th2 response, strong CD4+ T cell responses, and a low ratio of antigen-specific IgG4 to IgE following exposure [66]. Most of the IgE produced is not antigen specific but the killing of the parasite is mainly dependent on IgE, IgM, and the complement but also on other isotypes of specific antibodies [66-68]. Above all, there is an immediate type-2 cytokines production from both CD4+ T cells and other sources, within 24 hours of entry of L3 larvae into the host [69]. Studies have demonstrated that PBMCs from *W. bancrofti*-exposed endemic normal donors proliferated strongly, and produced high levels of IFN-γ, IL-2, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF) in response to stimulation with filarial antigen but no IL-4 could be detected in this study [70]. Thus endemic normals display Th1 response, observed with CP patients, and Th2 immune response associated with asymptomatic individuals; but have no known immunoregulation mechanisms. Figure 4 represents the spectrum of clinical outcomes observed in filarial infections.

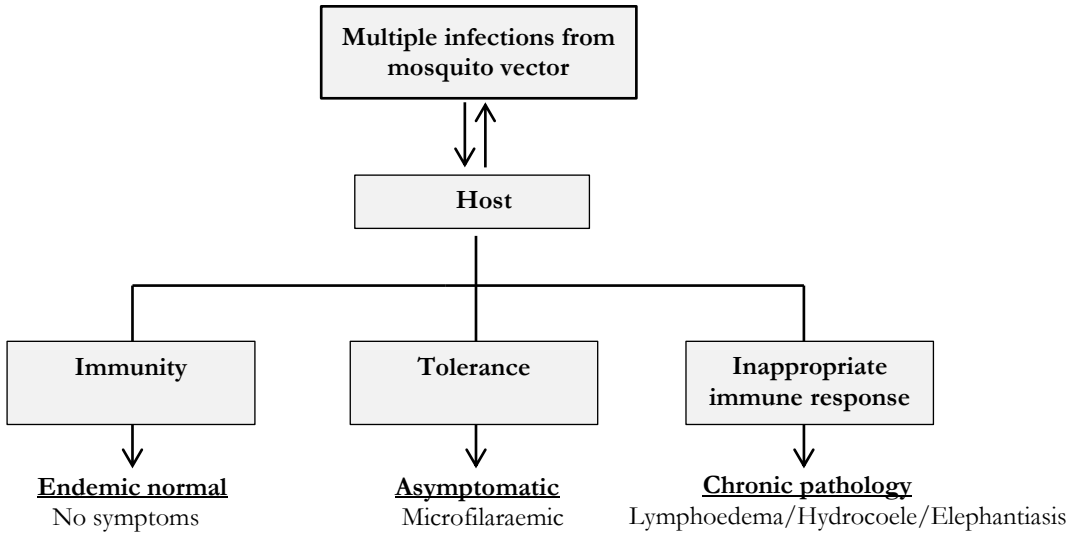

Figure 4: Spectrum of clinical outcomes in infection with filarial worms. Figure represents the different phenotypes of the immune response in lymphatic filariasis endemic regions and the associated clinical manifestations. Adapted from [71] and [72].

1.2.2. Immunoregulation by filarial worms

During filarial infection, the immune system is exposed to parasite-derived molecules, including proteins, lipids, present either at the surface of the worms or in the excretory-secretory (ES) products [73]. Interaction of these molecules with host cells can result in a shift of the immune response, from an inflammatory towards an anti-inflammatory type of response. This shift is the result of several mechanisms used by the parasites to avoid their destruction by host-mediated immune response. These mechanisms include mainly the suppression of host immune molecules by parasite-produced homologue products and the regulation of inflammatory pathways [73]. The suppression of host molecules and immune pathways by parasite-released products has been extensively studied and depends on the form of molecular recognition between parasite and host. Filarial parasites may secrete host mammalian cytokine homologues such as TGF-β that were shown to bind the human TGF-β receptor and influence Treg development [74,75]. Immune "non-responsiveness" may also be the result of deactivation of immune molecules or factors by parasitic products such as macrophage migration inhibitory factor (MIF) [76]. While mammalian MIF has numerous functions and acts in particular as a pro-inflammatory cytokine, *B. malayi* MIF has direct chemotactic effects on human monocytes but appears to be associated with anti-inflammatory modified Th2-type responses [77,78]. Also *B. malayi* Calreticulin, a protein from both adult worm and L3 larvae, has been reported to prevent complement activation via interaction with complement first component C1q [79]. In LF, most of filarial-derived proteins with host immunoregulatory properties have been discovered with *Brugia malayi*, little is known about *Wuchereria bancrofti* and *Brugia timori*.Filarial worms-derived products are also able to modulate the function of non-immune and immune cells [80]. From the beginning of infection, down regulation of innate response may occur. Typically expanded populations of eosinophils, basophils, mast cells and macrophages appear [53,81,82]. These cells are potent

to impair antigen-specific T cell responses [83]. They can modify dendritic cells (DCs) function and downregulate adaptive immune responses, through the induction of a regulatory network that target lymphocytes T and B as well as macrophages.

DCs are the main messenger cells to communicate with T cells and initiate an immune response, interference with their functions represents a key mechanism for the parasites to impair immune response and last their survival [83]. Filarial-derived products may influence antigen-presenting process by modulating DCs functions [84]. Nitric oxide (NO), produced by activated macrophages, eosinophils and other myeloid cells, is involved in many signaling pathways and may mediate induction of immunosuppression [85]. NO production is associated with depletion of lymphocyte subpopulations and impaired function of antigen-presenting cells, such as DCs [86]. Toll-like receptor (TLR) responsiveness, expression of costimulatory molecules and production of pro-inflammatory cytokines in DCs are suppressed in filarial infections, leading to an impaired ability of dendritic cells to produce IFNγ, MIP-1, IL-12, and IL-1 in response to TLR ligands [83,87-90]. In presence of *B. malayi* microfilariae, human DCs showed higher levels of apoptosis and decreased production of IL-12 and IL-10 [91,92].

Macrophages are frequently the most abundant cell type recruited to the site of helminth infection but their activation and role are strictly dependent on the parasite, the stage of infection and localization of the parasite. Parasites induce regulatory effectors like CTLA-4, PD-1 and ICOS and produce protease inhibitors that are capable of blocking peptide antigen presentation and of eliciting an IL-10 response from macrophages. Indeed blocking CTLA-4 or neutralizing TGF-β restored the ability to mount Th1/Th2 responses to live parasites and reversed the induction of anergy-inducing factors [61]. In addition, filariae cystatin, a well-characterized protease inhibitor, exploits host signaling events to regulate cytokine production in macrophages [93]. Macrophages that are activated by the Th2-type cytokines IL-4 and IL-

13 develop an alternatively activated phenotype (AAM), the most active cell populations in regulation of immune response [84]. In most Mf+ individuals, suppression of inflammation is propagated by AAM as anti-inflammatory down-regulatory cells [94,95]. These cells are sources of TGF-β and IL-10 [96,97] as well as prostaglandins PGE2 [98] and the IL-1 receptor antagonist [99], leading to immunomodulation of APCs. However, AAM are also involved in repairing tissue or wound healing followed migration of larvae through the host tissue. In human filariasis, alternatively activated macrophage markers are up-regulated in the blood of asymptomatic microfilaremics, the category displaying T cell hyporesponsiveness [100].

T cells hyporesponsiveness to antigen-specific stimuli from the beginning of infection may support survival of the developing stages of the parasite [101,102]. Induced hyporesponsiveness of T cells as a defect in lymphocyte function may contribute to the failure of the immune system to eliminate filarial nematodes. Filarial infections induce both natural and adaptive Treg cells expressing the Foxp3 transcription factor in the host and secreting high levels of IL-10 and TGF-β [103,104]. Thus these regulatory T cells can alter the course of inflammatory disorders by increased production of IL-10 and TGF-β. Furthermore this inhibits production of IL-12 by DCs, thus suppressing Th1 responses [105]. However *in vitro* neutralization of IL-10 and TGF-β, at least partially, restores T cell proliferation and cytokine production in lymphatic filariasis [72,106,107]. Moreover, increased expression of CTLA-4 and PD-1 has been demonstrated to be involved in hyporesponsiveness observed in cells from infected individuals [65,108]. Recently, Babu et al. demonstrated that regulatory T cells from microfilaremic individuals, but not those from uninfected individuals, suppress both Th1 and Th2 cytokines production, providing further evidence of a link between Tregs and the immunosuppressive state [109] .

The immunosuppressive effect may be also maintained by other mechanisms such as induction of immunosuppressive B cells and regulatory function in filarial infection is also pointed for B cells. IL-10 and TGF-β are secreted form B cells during Brugian infection [110]. The Th2 response which controls B-cell class switching to both IgG4 and IgE, requires IL-4 or IL-13 cytokines. In asymptomatic microfilaraemic patients, the Th2 response is strongly correlated with the expression of immunoregulatory cytokines like IL-10 and TGF-β. This particular immunosuppressive environment is associated with reduction of IgE antibody production by B cell and induction of high plasma levels of IgG4 [29,111].

Thus immunosuppressive action of filarial parasites is primarily directed to antigen-presenting cells (APC) and induction of regulatory T, B cells and macrophages, with the common effect to selectively inhibit local or systemic immune cells including granulocytes.

1.3. Granulocytes and degranulation in filariasis

1.3.1. General features of granulocyte-mediated protection in LF

Granulocytes, the collective name given to neutrophil, eosinophil and basophil leucocytes, play a prominent role in immune defense. They are key effector cells at the frontline against infections with filarial worms [112,113]. Under normal conditions, the human blood contains up to 50% neutrophils; 1-5% eosinophils and less than 1% basophils of circulating blood leukocytes [113]. Depending on the specific context, granulocytes may have pivotal roles in host protection, immunopathology, or facilitation of helminth establishment. Figure 5 depicts the implication of granulocytes in host protection against helminths. During helminth infection, granulocytes are rapidly activated and recruited to sites of infection where they are key producers of cytokines such as IL-4 and IL-13, enhancing Th2 responses [112,114,115].

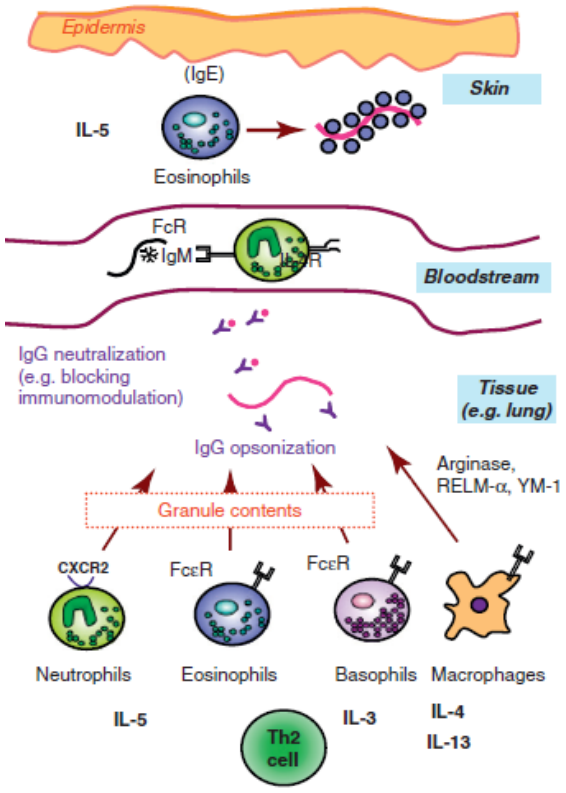

Figure 5: Protective role of granulocytes during helminth infection. Figure represents the different mechanisms by which granulocytes attack and clear skin-penetrating helminth larvae, bloodstream microfilariae and tissue-dwelling helminth worms. Obtained from [65]

Upon activation they can also release "alarmins" which are constitutively available endogenous molecules, such as defensins, cathelicidins, high-mobility group box protein 1 and the RNAse eosinophil-derived neurotoxin, that act as chemo-attractants while providing maturation signals to antigen-presenting cells such as dendritic cells (DCs) and macrophages [80,116]. The activation of granulocytes can be measured *in vitro* by monitoring the expression of several activation markers including mainly CD63, a member of the tetraspan membrane glycoprotein family [117,118]. CD63 is an activation marker specific for neutrophils and basophils and, among other markers, for eosinophils [117,119,120]; and it is responsible for the retention and sorting of pro-neutrophil elastase in the primary granules of neutrophils [117,119].

Once activated, granulocytes can attack filarial worms through antibody-dependent cell mediated cytotoxicity (ADCC), which implies the killing of antibody-coated parasites via the release of cytotoxic granules (degranulation). The release of granule proteins can be induced through several cytokines, mainly IL-3 and IL-5 [121,122], and through binding of IgE/IgG-bound antigen complexes to the high affinity IgE (FcεRI) and IgG (FcγRI), receptors that trigger a tightly controlled kinases phosphorylation cascade including Src, SHIP-1, SHP-1, PI3K and Syk [123,124]. Human granulocytes express FcγRI, FcγRIIa/b, FcγRIII, FcεRI/II and FcαR. Upon stimulation, complete granule contents are released by fusion with the cellular membrane and cytolysis. Six major granule proteins are known for granulocytes: major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), neutrophil elastase (NE) and histamine. MBP, EPO, and ECP are potent helminth toxins, which implicates granulocytes as key effector cells against helminths' invasion. However, emerging data in mice models pointed out the possible role of granulocyte eosinophils in helminth establishment [125,126]. During human helminthic infection, the function of granulocytes as effector cells has been difficult to study. Furthermore, the short life span and the diverse and conflicting roles of granulocyte subpopulations limited the manipulation and investigation of these cells.

1.3.2. Neutrophils

Tissue invasion by filarial worms initiates an acute inflammatory response leading to rapid neutrophil recruitment. At the site of filarial worm infection, neutrophils are characterized by their ability to act as phagocytic cells, to release anti-helminthic factors and to produce signal mediators for other immune cells. Neutrophils are the main granulocytes efficient at phagocytosis and they can engulf and kill pathogens in phagolysosomes by generation of oxidative (with reactive oxygen species) and non-oxidative mechanisms. Moreover recent study suggested that neutrophils may play a non-phagocytic role in the transfer of pathogen to

local lymph nodes in antigen presentation and in early T-cell recruitment [127]. However, helminths are too large for phagocytosis and as consequence a neutrophil will then disgorge cytotoxic granule contents and immune mediators into the surrounding environment causing tissue damage and amplifying the inflammatory response by signaling larger-scale phagocytes such as macrophages, dendritic cells and epithelial cells to initiate a phagocytic response. Neutrophils contain, at least, four different types of granule: primary (azurophilic), such as neutrophil elastase and defensins, secondary (specific), tertiary (gelatinase) and secretory. These granule contents are potent inducers of parasite killing [128,129]. In addition to their toxic effect, the granule contents and other released neutrophil factors are well known as immune mediators. These products may act in conjunction with cells resident in the affected tissue, such as macrophages and mast cells, to induce the recruitment of additional neutrophils and other leukocyte populations (monocytes, lymphocytes, eosinophils and basophils). Chen *et al.* describe a previously unknown and unexpected role for neutrophils in 'training' macrophages, upon IL-13 production, to acquire a long-term protective function against helminth larvae as they transit through the lungs [130]. Furthermore, neutrophils are important mediators of the Th17 pathway of resistance to pathogens [131]. However, excessive production of toxic proteins and activation of cellular defenses may cause inappropriate damage to host tissues linked to the pathogenesis of a variety of pulmonary diseases and of LF. Nonetheless, the systemic leukocytosis that accompanies AFL episodes in LF is shown to be dominated by eosinophils rather than neutrophils [132].

1.3.3. Eosinophils

Under normal circumstances, eosinophils account for less than 5% of the circulating leucocyte population. However, during intense helminth infection the proportion of peripheral blood eosinophils can reach 40% under the influence of Th2 cell-derived IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [133]. Eosinophils are

recruited to the site of infection by the chemokines eotaxin-1 (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26), which bind to the receptor CCR3 [134-136]. Once accumulated in filarial worm-infected tissues, eosinophils are activated by cytokines (among others, IL-3 and IL-5) through receptor-mediated signals. Activated eosinophils act as effector cells by being involved in the killing of filarial parasites, particularly those with tissue-migratory larval stages, and as modulator of immune responses by releasing cytokines and chemokines [137]. In contrast to neutrophils, eosinophils have limited capacity of phagocytosis. The mechanism by which eosinophils mediate killing of filarial parasites, involves mainly antibody-induced release (ADCC), complement-induced release (CDC), or both of toxic granule proteins and reactive oxygen intermediates by activated eosinophils. However, evidence suggests that there might be significant differences in eosinophil-mediated killing mechanisms between different life-cycle stages of the same parasite [138,139]. Like neutrophils, eosinophils are supplied with numerous granules including MBP, ECP, EPO and EDN. The release of these secondary granule proteins, upon immunological stimuli and LF worm-derived excretory-secretory products, may directly damage tissues, infectious Mf or worms. *In vitro* the eosinophil granule proteins MBP, EPO, EDN and ECP have all been shown to kill *Brugia spp.* microfilariae [140]. In *in vivo* filarial-mouse models, the requirement of the granule proteins EPO and MBP in clearance of parasites depends upon the parasite species, the parasite stage and whether the response is a primary or a secondary infection. In models of brugian filariasis, eosinophils are necessary for Mf killing during primary but not secondary infections [141,142], where they are known to play a more immunomodulatory role. Interestingly eosinophils have been suggested to influence the immune response in a manner that would sustain chronic infection and insure worm survival [125]. Thus in eosinophil-deficient mice infected with *T. spiralis*, larval killing was enhanced [125] whereas adult worms of *L.*

sigmodontis exhibited an accelerated growth in response to a vigorous eosinophil response during larval entry [126].

Besides these peripheral effector functions, eosinophils are modulators of immune responses. They play an early role in innate immunity by production of important cytokines that modulate adaptive immunity. Recent studies have demonstrated that eosinophils can process and present a variety of parasitic antigens and, by doing so, function as antigen-presenting cells [143,144]. Eosinophils and the granule proteins they release have recently been shown to induce migration and maturation of DCs, as well as stimulating enhanced levels of IL-5, IL-6, IL-10 and IL-13 cytokines and IgG1 and IgG2 antibodies and thereby amplifying Th1 and preferentially Th2 immune responses [145]. Eosinophils are the main cell population involved in the development of TPE [146]. They are found in late-phase allergic reactions; characteristic they share with basophils.

1.3.4. Basophils

Basophils are odd polymorphonuclear granulocytes poised at the interface between the innate sensing of antigens and the initiation and execution of adaptive Th2 cytokine responses. Following helminth infection, basophils increase in number in the blood and tissues. They are rapidly mobilized and can be efficiently recruited into lymphoid and peripheral tissues where they execute their effector functions. It has been proposed that IL-4-producing cells of the innate immune system such as mast cells, eosinophils or basophils might provide the initial source of IL-4 to drive T cell polarization toward Th2 cells. T cell-derived IL-3 seems to play a major role in helminth-induced human basophil activation [147,148]. IL-3 might not only mobilize basophils from the bone marrow but also increase their survival [148,149]. It could be further demonstrated that IL-3 induced release of IL-4 from basophils [150]. Helminth extracts have also been shown to induce release of IL-4 but also IL-5 and IL-13 from human and murine basophils in the presence of IgE [151,152]. Basophils can be activated through an

IgE-dependent or IgE-independent process, secreting important amounts of IL-4 as well as mediating degranulation and releasing chemotaxis and mediators such as histamine. Basophils are characterized by the presence of basophilic granules and surface expression of high affinity FcεRI for binding of IgE, in addition to cytokine, chemokine and complement receptors. Upon ligation they release chemical mediators and particularly Th2 cytokines, which implicates basophils in immune responses elicited by helminths. In addition to that, studies have demonstrated that depletion of basophils results in impaired protection against several gastrointestinal helminths [151,153]. In murine helminth infection, basophils have recently been shown to process antigen and stimulate naïve CD4 T cells in peripheral lymphoid tissues [153-155]. Despite this induction of an immune response, basophils may not be implicated in protective responses during primary helminth infection [156,157]. However, they may play a major role in type 2-mediated secondary infection in conjunction with CD4+ T cells, as depletion of IL-4 and IL-13 in both basophils and CD4+ T cells was necessary to abrogate protection [157]. Moreover, chronic helminth infection reduced basophil responsiveness in an IL-10–dependent manner [158]. Like neutrophils and eosinophils, basophils are implicated in a spectrum of diseases. Basophils display a remarkable potential to contribute to the symptoms of allergic inflammation through the release of histamine and leukotrienes. They have been proposed to play a key role in class switching to IgE in B cells [159] and thus in antibodies production.

1.4. Antibodies in LF

1.4.1. Important role of IgG/IgE antibodies in host protection

Filarial worms' invasion and subsequent presentation of filarial worm-associated antigens to T cells lead to cytokine production and innate and adaptive cells mobilization. The cytokines IL-4 and IL-13 induce antigen-specific B cells differentiation and production of large amounts

of antibodies. The isotype and titer of antibody produced play a crucial role in protection against nematode infections [52]. Vaccination increases specific antibodies production by host. These immunoglobulins play diverse roles in parasite expulsion and establishment. Several studies demonstrated the preliminary role of IgM antibodies in Mf clearance [160,161] and complement cascade activation [52,162]. As the role of IgM are limited, other isotype antibodies such as IgE and IgG subclass antibodies contribute to filarial immunity.

IgE production is crucial for the elimination of parasites. IgE stimulates immune cells and this results in activation of the granulocytic cells; mast cell, neutrophil, eosinophil and basophil via receptor binding [52]. FcεRI is the IgE receptor on granulocytes that is involved in allergic reactions and defense against parasitic infections. The interaction of FcεRI with the Fc portion of helminth-bound IgE causes the granulocytes to release granule contained proteins in a mechanism similar to that of the NK cell during ADCC. Cellular degranulation releases several mediators that lyse and destroy parasites [52,163]. Released mediators may also serve as chemoattractant for other immune cells and thus amplify immune responses. Functional activity of IgE antibody depends on its interaction with receptor on effector cells and a particular receptor can lead to either protective immunity or immunopathology. An excess of IgE production results in serious allergic reactions including anaphylactic shock and TPE.

Host protection and regulation by IgG antibodies and B cells is recognized as an essential component of the Th2 response in helminth infections [164]. IgG was identified as the antibody isotype that provides the most effective protective immunity against *H. polygyrus bakeri* [165]. Jankovic and colleagues demonstrated in a murine model of acute *S. mansoni* infection, where the dominant isotypes are IgG1 and IgE [164], that mice deficient in B cells are unable to downregulate granuloma formation in chronic infection [166]. However, this is mediated by the Fcγ receptors, which indicates a role for IgG antibodies in the down-modulation of pathology [166]. In mice with Strongyloides infection, IgG antibodies are

shown to play an essential role in the killing of compartmented larvae housed in diffusion chambers implanted subcutaneously into mice for a 24 hour period that allow transfer of only serum and cells [167-169]. Several studies evoked in LF infections the strong filarial antigen specific IgG responses in infected or exposed individuals, who have expelled LF infections compared to uninfected non-endemic persons [170-172]. These findings are supported by observations of passive transfer of immunity, against helminthes, to naive experimental animals using immune serum or purified IgG. Passive immunity has been shown using IgG monoclonal antibodies specific for *Fasciola hepatica* [173] and *S. mansoni* [174] and IgG or IgA antibodies specific for *T. spiralis* [175,176]. In addition parasite-specific maternal IgG have been reported to protect neonates against infection with the helminthes *T. spiralis* [177] or *H. polygyrus bakeri* [178]. These data indicate that antibodies, particularly IgG, can act as potent mediators of protective immunity following helminth infections. The IgG-mediated protective immunity is achieved via different mechanisms.

Anti-filarial IgG antibodies opsonise helminths and by binding to Fc receptors (FcR) and activating NK cells, mast cells, eosinophils or neutrophils and, in turn, orchestrate the killing of the parasite by antibody-dependent cellular cytotoxicity (ADCC). ADCC is the mechanism by which antibody coated to helminths binds Fc receptors (FcR) on the effector cell surface and this initiates cell degranulation and extrusion of toxic granule contents onto parasites. The engagement of immune effector mechanisms including ADCC, ADCP (antibody-dependent cellular phagocytosis) and CDC (complement-dependent cytotoxicity) is highly dependent on the interaction of the IgG Fc domain with FcγR on effector cells. Fc-mediated effector functions are especially important against filarial infections where cellular and complement mediated responses are important for efficient parasite clearance. These FcγR can be both activating (FcγRI, FcγRIIa and FcγRIIIa) and inhibitory (FcγRIIb). IgG antibodies binding on these receptors signal through immunoreceptor tyrosine-based activating motifs (ITAM) or

inhibitory motifs (ITIM) to elicit or inhibit immune functions. Importantly the interaction of IgG with FcγRIIb activates the associated intracellular domain bearing ITIM characterized by the recruitment of SH2 domain-containing phosphatases such SHIP1, which inhibits ITAM-dependent kinases cascade pathways including PI3K, Src, Syk [179-182]. However IgG-induced receptor activities can be altered by IgG post-translational modifications such as galactosylation, fucosylation and sialylation. But the biological role and regulation of each subclass of IgG in helminth infections are poorly known. They will be discussed separately.

1.4.2. IgG1

IgG1 is the most abundant subclass under normal conditions [111]. Following filarial exposure, IgG1 is produced prior to anti-filarial IgG4 [183,184]. The cytokines IL-4 and IL-13 act on B cells and induce expression of both IgG1 and IgE in mice and IgG4 and IgE in humans [185,186]. Higher expression of filaria-specific IgG1 and IgG2 has been correlated with the presence of microfilariae, irrespective of the presence or absence of adult worms [187]. Negative associations have been reported between IgG1, IgG2, and IgE antibodies and Mf status and positive correlations between IgG4 levels and active filarial infection have also been reported [187,188]. However, during filarial infections, the expression of IgG1 varies according to the gender. Punkosdy et al. demonstrated that women with lymphedema had a significantly higher median antifilarial IgG1 level than asymptomatic microfilaria positive and negative women [189]. In contrast, men with hydrocele who were microfilaria positive had a significantly lower median antifilarial IgG1 response and a significantly higher median antifilarial IgG4 response than men with hydrocele who were microfilaria negative [189], suggesting a role of IgG1 in the pathology of LF. The main role of IgG1 in host protection against filarial infections is to trigger ADCC beside its involvement in complement activation and immune cell expansion [111,162]. Following *H. polygyrus* infection, IgG1 and IgE have been observed to promote the expansion of basophils, which are known to be protective

during various helminth infections [190], indication IgG1 implication in protective immunity. Study demonstrated that vaccine-induced immunity to intestinal helminths involves IgG1 antibodies directed against secreted proteins [191]. However post-translational modifications modulate IgG1 immune functions. Consistently, highly galactosylated IgG1 has been shown to promote cooperative signaling of FcγRIIb with dectin-1, resulting in anti-inflammatory effects [192]. IgG1 glycosylations are reported to increase its affinity for the inhibitory FcγRIIb than toward the activating FcγRs, and can thus induce a modified activation threshold in innate immune cells that express both types of receptors [193,194].

1.4.3. IgG2

The role of this antibody in protective immunity is not yet fully understood. IgG2 is known to weakly bind complement and monocyte [52,111]. IgG2 was shown to bind to FcγRI on the surface of macrophages and to drive macrophage activation in an efficient trapping of tissue-migrating helminth larvae *in vitro* [195]. In LF, preliminary studies demonstrated that elevated levels were similar between asymptomatic microfilaraemics and patients with chronic pathology [172]. These results conflicted with other studies showing an inverse relationship between IgG2 levels and CFA [196]. In addition, analysis of IgG subclasses against Brugian antigen revealed a significant increase in IgG1 and IgG2 antibodies in the sera of endemic normals compared with microfilaraemic and non-endemic normal groups [170]. Further investigations are required to unveil the genuine role of IgG2 antibodies during helminth infections.

1.4.4. IgG3

IgG3 antibodies are particularly effective in the induction of effector functions including mainly ADCC and complement activation [52,162]. It is a potent pro-inflammatory antibody which, in some cases, can trigger excessive complement activation and thereby causes

damaging effect on the lymphatic vessels, leading to limb pathology in LF [163]. IgG3 has a shorter half-life; this may function to limit the potential of its excessive inflammatory responses. IgG3 was also shown to enhance antibody responses [197]. IgG3-mediated enhancement of antibody responses is not FcγR-dependent [197] and, interestingly, unlike IgE and IgG2, IgG3 did not induce proliferation of specific T cells *in vivo* [198]. IgG3's activity depends on the complement system [197]. In LF infections, significantly higher levels of IgG3, compared to other isotypes, have been observed in patients with elephantiasis and chronic pathology [163,172], indicating the role of this isotype in inflammation and limb pathology. Moreover, lower levels of IgG3 have been observed in individuals with active filarial infection than subjects free of established infection, in contrast to higher levels of filaria-specific IgG4 [187,199].

1.4.5. IgG4

IgG4 is the least abundant subclass of IgG in normal human serum, representing up to 4% of all IgG [52]. Elevated IgG4 levels are triggered in response to a chronic antigenic stimulus and inflammation and are generally associated with states of immune tolerance. Increased production of IgG4 and IgE antibodies are commonly associated with filarial infections [29,66,200]. Filarial parasites are well known to induce, alongside the classical Th2 response, a strong regulatory response associated to Tregs, AAM and anti-inflammatory cytokines such as IL-10 and TGF-β. This type of response is commonly termed "modified Th2 response". The main feature of this modified Th2 phenotype is the inhibition of B-cell class switching to IgE and induction of high plasma levels of antigen specific IgG4 antibody [29,111].

IgG4 is structurally and functionally different from its co-class members. While IgG1, IgG2 and IgG3 are able to fixe and activate complement, IgG4 has no affinity for the complement and can even, as demonstrated by van der Zee et al., in a phospholipase-A model, inhibit

complement activation by other antibodies [201]; therefore IgG4 is unable to activate protective immune mechanisms implicating the complement. Furthermore, in contrast to IgG1 and IgG3, IgG4 cannot induce antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis after binding FcγR on the surface of immune cells [111,202,203]. The effect induced by IgG4 in host immune cell after interaction with FcγR is poorly understood. IgG4 was demonstrated to bind to FcγRI, FcγRIIa/b and FcγRIIIa receptors with different affinity [203-206]. To complicate the situation, IgG4 was shown to inhibit IgE-mediated basophil activation after cross-linking of the inhibitory receptor FcγRIIb and the IgE high affinity receptor FcεRI [207]. Moreover, Karagiannis et al. found that IgG4 promoted by tumor-induced Th2 inflammation compete with IgG1 for FcγRI and inhibited IgG1-mediated tumoricidal functions [203]. In addition, IgG4 is also known to compete with IgE for the antibody fixation sites on mast cells, eosinophils [208,209] and basophils [210-212] and thus impaired cell degranulation. In these studies, IgG4, unlike IgE, cannot cross-link receptors on basophils, mast cells, or eosinophils. These anti-inflammatory properties of IgG4 antibodies are associated with its unique ability to undergo the "so called" Fab-arm exchange (FAE); resulting in the creation of bispecific, functionally monovalent antibodies with limited capacity of immune complex formation. IgG4, thus, appears to be a canonical marker of the modified Th2 state, potentially blocking cytophilic isotypes such as IgE and preempting potentially damaging inflammation [62]. Indeed, IgG4 isotype can be both beneficial and detrimental to the host, depending on titre and ratio to IgE. In LF, the ratio IgG4 to IgE is crucial since higher ratios have been associated with microfilaraemia and the inverse with tropical pulmonary eosinophilia [213]. High levels of IgG4 but low levels of IgE are found in the blood of filaria-infected hyporesponsive, asymptomatic persons [29,214]. Thus IgG4 may prevent immunopathological responses in asymptomatically infected individuals. Several studies demonstrated that the titre of IgG4 antibodies correlated with the hyporesponsive state

and the decreased rate of allergy and autoimmune diseases observed in Mf+ individuals, whereas IgE, IgG1 and IgG3 are linked with chronic pathology [29,64,215,216]. Interestingly, cell hyporesponsiveness can be reversed by the chemotherapeutic removal of the parasite burden and strong correlation has been suggested between IgG4 levels and unresponsiveness [217-219]. Thus, extremely high IgG4 levels in many helminth-infected patients reflect a dominant regulatory environment. Therefore, the induction of IgG4 in the asymptomatic individuals seems to represent one major mechanism used by filarial parasites to evade destruction by host's immune effector mechanisms [29]. But how IgG4 down-modulates effector mechanism such as granulocyte activation and degranulation is still unclear. The main goal of the present thesis is to address this question. In addition, since recent studies reported that inhibition of complement activation might be a possible mechanism of parasite-induced immunomodulation [220-222]; this thesis investigated the role of IgG4 antibodies in complement inhibition.

1.5. Complement system – major innate component in LF

The complement system is an important and fundamental part of the immune system, providing protection against pathogens by innate as well as by antibody-mediated immunity. It is a central component of the innate immune response that fulfils numerous functions including the recognition of foreign cells, communication with and activation of Th1 and Th2 responses and the removal of cellular debris [52,223,224]. Thus it acts as a bridge between the innate and adaptive immune systems. The human complement system consists of over thirty circulatory or membrane-bound plasma proteins and is activated upon detection of pathogen-associated molecular patterns or danger-associated molecular patterns. Once cleaved during the activation step, these proteins participate in different cascades, consisting of receptors and soluble proteins. They interact with one another and also with other cell receptors promoting

elimination of antigens, parasites and immune complexes, thereby regulating the immune response [225].

1.5.1. Complement activation pathways

The complement system can be activated at the site of infection by three main different pathways which include the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP). Each pathway, with its own recognition molecules that initiate activation, converges in the activation of the central component C3 that leads to the formation of a membrane attack complex and lysis of the parasites.

1.5.1.1. The classical pathway

The classical pathway is initiated when C1q binds either directly to bacterial/parasite surface components or indirectly to the Fc portion on antigen-bound antibodies containing IgM or certain IgG subclasses [226]. The binding of C1q to foreigner antigens or immune complexes and the consequent activation of C1s–C1r–C1r–C1s tetramer cleave C4 into C4a and C4b. C4a, the smaller peptide fragment is released in the fluid phase as an anaphylatoxins whereas the larger fragment C4b binds covalently to the activator cell surface via its thioester group [227]. C4b will then bind to C2 component C2a forming C4b2a, the enzymatically active part of the classical pathway. As the complement cascade progresses, C4b2a proteolytically cleaves C3 into C3a and C3b. C3a promotes pro-inflammatory cellular response while C3b acts as an opsonin that binds to the surface of parasites and helps in phagocytosis. C3b bound to the C3 convertase will lead to the formation of C5 convertase that cleaves C5 into two fragments C5a and C5b. Finally, C5b initiates the activation of the terminal complement activation cascade (C6-9) leading to the formation of the membrane attack complex (MAC) by covalent binding to the parasite surface and to the lysis of the parasite. Rapid clearance of immune complexes, dying cells and debris from damaged tissues is the classical function that

is performed efficiently through activation of the classical pathway. Primary deficiency of C1q, C1r, C1s or C4 is closely linked to development of systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), thought to be due in part to the inability of complement to clear immune complexes and dying cells [228,229].

1.5.1.2. The lectin pathway

In contrast to the classical pathway, the lectin pathway neutralizes the invading microorganisms through a rather selective and an antibody-independent mechanism. The lectin pathway is initiated when circulating mannan-binding lectin (MBL) or ficolins bind to patterns of carbohydrates (pathogen-associated molecular patterns (PAMPs)) present on pathogens or damaged self, activating MBL-associated serine proteases (MASP) to cleave C2 and C4 components. The C4b fragment (product of C4 cleavage) binds to the pathogen surface and associates with C2a to form the C4b2a (similar to the C3-convertase of the classical pathway). Once C3 cleaved, the C3b fragment can bind to the pathogen surface to activate the alternative pathway (section 1.5.1.3), or it can bind to the C4b2a (lectin pathway C3-convertase) to form the C5- convertase (C4b2a3b). C3b can also bind to the alternative pathway C3-convertase and form the C5-convertase. Then, the C5-convertase initiates the formation of the terminal pathway as described before for the classical pathway. Although low MBL is common in human populations, it was thought that deficiency of MBL might explain some cases of increased susceptibility to bacterial infection [230].

1.5.1.3. The alternative pathway

In contrast to the specific protein-protein or protein-carbohydrate interactions that characterize classical and lectin pathway activation, the alternative pathway activation occurs through auto-activation at low levels, independent of a large multivalent recognition molecule. Factor B, factor D and factor P (properdin) are specific components of the

alternative pathway of complement activation [231]. Slow spontaneous hydrolysis of the thioester bond of C3 generates C3(H_2O), which in the presence of factors B forms C3(H_2O)B, cleaved by factor D into two fragments Ba and Bb. The Bb fragment form with C3(H_2O) the initial alternative pathway C3 convertase, the C3(H_2O)Bb.. C3bBb, resulting from components C3b and Bb, cleaves additional C3 molecules, generating more surface-bound C3b. Through this positive feedback loop, the alternative pathway amplifies complement activation, even when C3b is initially produced by other pathways (Figure 6).

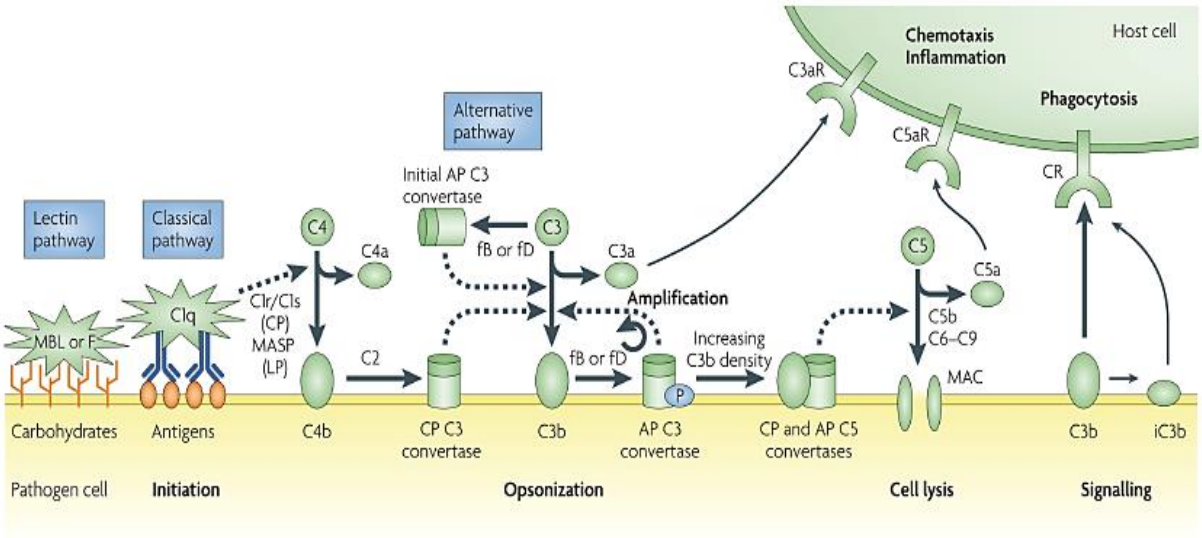

Figure 6: Main pathways of complement activation. Figure shows the activation of the complement by antibody complexes (classical pathway (CP)), terminal mannose (lectin pathway (LP)) or by spontaneous and induced C3 hydrolysis (alternative pathway (AP)). The different pathways culminate with the formation of C3 convertases which activate C3b. Covalent binding of C3b (opsonization) amplifies the cascade and mediates phagocytosis and immune responses by binding to complement receptors (CR). C3b also leads to the activation of C5b which initiates the formation of the lytic membrane-attack complex (MAC), meanwhile C3a and C5a induce pro-inflammatory and chemotactic responses by binding to their receptors (C3aR and C5aR) on cell surface... Adapted from [232]. fB: factor B; fD: factor D; fI: factor I; MASP: Mannose-binding lectin-associated serine protease.

This loop of successive proteolytic steps is enhanced by the serum protein properdin, which stabilizes protein-protein interactions, and in turn instability of C3bBb, during the process.

Further accumulation of C3b fragments by the continuous activity of the C3 convertase C3bBb complex shifts the specificity of this complex towards the cleavage of the substrate C5 and forms the alternative complement pathway C5 convertase complex. The C5 convertase cleaves the C5 component of the complement and thereby releases C5a and initiates the formation of the membrane attack complex. The serum protein properdin, secreted by monocytes, granulocytes and T cells [233,234], serves as a positive regulator of the alternative pathway of complement activation. Once activated, complement leads to a multiple of biological activities.

1.5.2. Complement functions and regulation during helminth infections

1.5.2.1. Biological functions of the complement

The biological functions of complement include opsonisation of the invading pathogens, initiation of a pro-inflammatory response, direct lysis of pathogens via the membrane attack complex and finally immune complex clearance [235]. Several immune cells express complement receptors on their surfaces. Many functions of the complement system are exerted through binding of complement derived fragments to these receptors. Upon complement activation, the initiating particle is covered with C3 and/or C4 fragments by opsonisation. These fragments are bound by complement receptors on phagocytes, which facilitate phagocytosis. The complement activation cascade produces three small fragments with pro-inflammatory effects: the anaphylatoxins C3a, C4a and C5a. These stimulate the release of mediators that increase vascular permeability for neutrophil recruitment [236]. Complement cascade proteins have been demonstrated in filarial infection to opsonise filariae and thereby facilitating parasite recognition by NK cells and macrophages leading to release of cytotoxic granules and lyse of filariae [52]. One of the major functions of complement cascade is direct killing of invading pathogen through the formation of the membrane attack

complex formation, which leads to the disruption of membrane functions creating an osmotic gradient which finally leads to cell lysis [237]. The complement system is involved in the stimulation and regulation of the adaptive immune system. This is supported by study on patients with deficiencies of the classical pathway, who exhibit low Ig levels and impaired specific immune responses [238,239]. Dendritic cell's maturation is increased by C1q binding and interactions between T cells and dendritic cells are influenced by C3 fragments [240,241]. Early studies associated complement as a factor, *in vitro*, in mediating adherence of myeloid cells to nematode parasites and subsequent larval killing with considerable differences in susceptibility between parasite species [242]. In infection with *Nippostrongylus brasiliensis*, both mice, deficient in the alternative pathway, and animals, refractory to complement activation in general, following C3 deficiency, had higher numbers of larvae in the lungs during primary infection, indicating a role for complement in parasite attrition during tissue migration [243]. Moreover, blocking of C5a receptor indicated an important role for anaphylatoxins in this process as reduced recruitment of both eosinophils and neutrophils to the skin during the early phase of infection was observed [243]. An important task for the complement system is contribution to waste disposal. Several complement components significantly facilitate the clearance of dead cells by a system involving phagocytes. Complement also plays a role in maintaining the solubility of and removing circulating immune complexes generated during the adaptive immune response and thus prevents inflammatory complement reaction by these complexes. Excessive complement activation on self-tissue has severe effects and can lead to the development of various diseases. So complement activation must be tightly regulated to avoid tissue damage.

1.5.2.2. Complement regulation during helminth infections

A number of complement regulatory proteins that tightly regulate the activation of complement are present in the organism. These proteins are fluid-phase regulatory proteins

such as C4 binding protein (C4bp), C1 inhibitor, Factor H and Serum Carboxypeptidase N (SCPN); and membrane-bound regulatory proteins including complement receptor 1 (CR1 or CD35), protectin (CD59), membrane cofactor protein (MCP) or CD46 and decay accelerating factor (DAF). Beside the host's machinery to ensure protection, parasites developed strategies over co-evolution with their host to regulate or escape destruction by complement system. These strategies include mainly: the recruitment or mimicking of complement regulators, the modulation or inhibition of complement proteins by direct interactions and the inactivation by enzymatic degradation [232]. Several studies reported the regulation of complement activation during helminth infections. *Onchocerca volvulus* and *Dirofilaria immitis* were shown to display loss of complement fixation and/or cellular adherence as they make the transition from L3 to L4 stages [138,244]. Meri et al. observed that the microfilariae of *Onchocerca volvulus* inactivated complement by binding factor H which, in the presence of factor I, promotes the cleavage of C3b, and restricts amplification of the alternative pathway [221]. Secreted products from *Trichinella canis* infective larvae degraded the deposition of C3 and adherence of eosinophils to *N. brasiliensis* [245]. Another strategy used by helminth parasites consists in producing active proteins that target host enzymes or pro-inflammatory factors. Thus, eotaxin, a proinflammatory chemotactic factor, is cleaved and inactivated by secreted metallo- and serine proteases from *Necator americanus* [246]. Interestingly paramyosin from *T. spiralis* and *S. mansoni* inhibited classical complement activation and membrane attack complex by binding to human complement C1q and C9 [220,222]. Furthermore, Rees-Roberts and colleagues demonstrated that secreted products of *Brugia malayi* microfilariae inhibited anaphylatoxin C5a-mediated granulocyte chemotaxis by a serine protease [247].

Thus, immune effector mechanisms such as complement activation and granulocyte degranulation are regulated by helminth-secreted and/or induced modulatory molecules

during helminth infection. Modulation of these effector mechanisms by IgG4 immunoglobulin has been reported in many disease models but a direct link between complement and granulocyte inhibition and IgG4 antibody during filarial infections is not yet clearly established.

2. AIM OF THE STUDY AND OBJECTIVES

Lymphatic filariasis (LF) is a major public health concern that affects millions of persons worldwide. These individuals are marginalized in their communities. To tackle the infection, the World Health Organization (WHO) launched the Global Programme to Eliminate Lymphatic Filariasis (GPELF). The Programme has two main components: 1) interrupting transmission of lymphatic filariasis through mass drug administration and 2) managing morbidity and preventing disability. To achieve these goals, a better understanding of the immunological phenomena associated to the pathogenesis of LF is needed, which will help to improve treatment strategies and thereby reduce transmission and in turn eliminate filarial infection. It is well admitted that the pattern of host immune response to the worms/or microfilariae plays a critical role in the pathogenesis of the disease. Interestingly, most affected individuals are relatively tolerant to filarial worms. This hyporesponsive state is characterized by downregulation of immune effector mechanisms such as complement activation and granulocyte degranulation [29,52,62,112,113,247-250] and associated with establishment of chronic infections. Previous investigations have linked increased level of the non-cytolytic antibody IgG4 to the hyporesponsive states in filarial infections [60-62]. Despite these studies, there is a paucity of information on whether and how IgG4 is involved in the pathophysiology of human filariasis. This thesis addresses these issues and thereby contributes to the understanding of immune regulation and clinical outcome in human filariasis. For this reason, the role of this antibody in disease pathogenesis was investigated by comparing the effect of plasma, IgG and, IgG4 fractions from the four clinical categories of individuals (EN, Mf+, Mf- and CP) on the activation and degranulation of granulocytes. In addition, the role of IgG4 antibody in the prevention of complement activation as well as the mechanisms underlying IgG4-mediated suppression was further assessed.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Samples, controls and ethics

Patients and endemic controls' samples were collected between 2008 and 2010 in the Nzema East District in the western region of Ghana endemic for LF. No other human filarial species were endemic in the region. Recruited individuals were part of a clinical trial (Registration: ISRCTN15216778) (http: www.filaria.eu/projects/epiaf.html) [200]. Written informed consent was obtained from all participants. Persons eligible for participation were male adults in good health, 18–60 years of age, with a minimum body weight of more than 40 kg and without any clinical condition requiring chronic medication. Exclusion criteria included abnormal hepatic and renal enzyme levels (γ-glutamyltransferase > 28 U/L, glutamyl pyruvic transaminase > 30 U/L, creatinine > 1.2 mg/100 mL) assessed by dipstick chemistry, alcohol, drug abuse, or antifilarial therapy in the past 10 months. Study participants were examined by a clinician using physical methods and a portable ultrasound machine (180 Plus; SonoSite, Bothell, WA) as described previously [251]. Ethical clearance was given by the Committee on Human Research Publication and Ethics at the University of Science and Technology in Kumasi, and the Ethics Committee at the University Hospital Bonn (Ethikkommission der Medizinischen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn). Microfilarial load was determined by microscopic examination of fingerprick night blood samples as published [251]. Subsequently, 10 mL of venous blood was collected from each eligible volunteer and plasma was taken, aliquoted, stored at -20°C and then transferred to liquid nitrogen until used.

Samples include EN, residing in the endemic region but free of infection (CFA-, Mf-, n=14), clinically asymptomatic microfilaraemic (CFA+, Mf+, n=14) and amicrofilaraemic (CFA+,

Mf-, n=14) subjects, positive for circulating filarial antigen and a group of chronic pathological individuals termed "CP" (n=14), negative for filarial antigen.

To investigate the mechanisms sustaining granulocyte modulation by IgG/IgG4 antibodies induced during LF infection, blood samples were collected from European non-endemic donors (NEC, n=14) and were kindly provided by the Institute for Experimental Haematology and Transfusion Medicine, University Hospital of Bonn, Germany. Granulocytes were isolated from the buffy coats and plasma and IgG antibodies from NEC were used as controls. Ethical approval was obtained from the University Hospital of Bonn ethics committee.

3.1.2. Plastic and glassware

All plastic and glassware equipment used in this study were supplied by either Eppendorf (Hamburg, Germany), Engelbrecht (Edermünde, Germany), Brand GmbH (Wertheim, Germany), Greiner (Frickenhausen, Germany), Becton Dickinson (Heidelberg, Germany), Nunc (Roskilde, Denmark), or BD Diagnostics (Franklin Lakes, USA).

3.1.3. *Brugia malayi* adult worm antigen extracts

Brugia malayi adult worms were obtained from NIAID Filariasis Research Reagent Resources Center (FR3) (University of Georgia, Athens, GA) and the antigen extracts prepared as previously described [66,252] (section 3.2.1.1).

3.1.4. Cytokines and recombinant proteins

Recombinants human rIL-3, human monoclonal anti-IgE antibody (used as part of the stimulus cocktail with *Bm*Ag) and recombinant human complement C1q were obtained respectively from Miltenyi Biotech (Bergisch Gladbach, Germany), Abnova (Taipei, Taiwan) and Sigma-Aldrich (Saint Louis, Missouri, USA).

3.1.5. Antibodies and purification matrix

Antibodies for flow cytometry

Conjugated antibodies used for flow cytometric characterization of granulocyte (anti-human CD66b-FITC (clone: G10F5)), granulocyte activation (anti-human CD63-PE (clone: H5C6), CD63-PE-Cy7 (clone: H5C6), HLADR-FITC (clone: LN3), HLADR-APC (clone: LN3)), granulocyte neutrophils (anti-human CD15-PE (clone: HI98), CD16-FITC (clone: CB16)), granulocyte eosinophils (anti-human CD11b-PE (clone: ICRF44), Siglec8-FITC (clone: 7C9)) and granulocyte basophils (anti-human CD203c-PE (clone: NP4D6), CD123-FITC (clone: 6H6)) were all obtained from Affymetrix eBioscience (San Diego, CA, USA). For the analysis of the phosphorylation of SHIP kinase, rabbit anti-human phospho-SHIP antibody (clone: 3635) was purchased from Stemcell Technologies (Köln, Germany) and FITC conjugated goat anti-rabbit secondary IgG antibody (clone: 6717) was from Abcam (Cambridge, UK).

For blocking assays

Anti-human CD64, CD32 and CD16 antibodies blocking respectively FcγRI, FcγRII and FcγRIII on the surface of granulocytes were purchased from Biolegend (San Diego, CA, USA).

For immunofluorescence

Unconjugated mouse anti-human IgG4 and Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) antibodies used for immunofluorescent analysis of IgG4 binding on granulocytes were obtained from Thermo Fisher Scientific (Rockford, USA).

For western blot

For western blot analysis of purified IgG/IgG4 fragments, unconjugated mouse anti-human IgG (H+L) and IgG4 antibodies and alkaline phosphatase-conjugated goat anti-mouse IgG antibody were respectively obtained from Thermo Fisher Scientific (Rockford, USA) and Bio-Rad Laboratories (Hercules, California, USA). To investigate IgG4-mediated intracellular signaling pathways, rabbit anti-human Src, Syk, SHIP antibodies and their respective phosphorylated forms (phospho-Src, Syk, SHIP) as well as anti-human β-actin and goat anti-rabbit IgG alkaline phosphatase-conjugated were purchased from Cell Signaling Technology (Beverly, USA).

Purification matrix

Prepacked Protein G matrix from GE Healthcare (Freiburg, Germany) and CaptureSelect Human IgG4 affinity matrix from Thermo Fisher Scientific (Rockford, USA) were used for IgG and IgG4 antibodies purification.

3.1.6. Software

ProcartaPlex Analyst software 1.0 was used for the Luminex bead array analysis of the levels of immunoglobulin isotypes in plasma and IgG/IgG4 fractions.

SoftMax Pro 5.4.1 was utilized for the assessment of granulocyte granules content release and analysis of C1q binding and activation.

BD-FACS-Diva analysis software was used for flow cytometric characterization of granulocytes, granulocyte subtypes and their activation.

Zen 2 software 1.0 (blue edition) was utilized for immunofluorescent IgG4 binding on granulocytes.

3.2. METHODS

3.2.1. Modulation of granulocyte activation and functions in LF

3.2.1.1. *Brugia malayi* adult worm antigen extracts preparation

Because *Wuchereria bancrofti* cannot be maintained in laboratory animals and are not easily obtained from human, *Brugia malayi* extracts were used and soluble antigen extracts were prepared from adult worms of the closely related human filarial parasite *Brugia malayi*. Adult worms were recovered from the peritoneal cavity of jirds (*Meriones unguiculatus*) experimentally infected with *B. malayi* infective larvae (L3) and washed extensively and homogenized with sterile PBS. The homogenate was sonicated and centrifuged at 300g for 10 minutes (min) at 4°C. Thereafter the protein concentration was determined using Bradford Assay (section 3.2.2.4) and the extracts were recovered, aliquoted and stored at -80°C until used. The extract was titrated to determine the optimal concentration for cells stimulation and the level of its endotoxin tested using the kinetic *Limulus amoebocyte* lysate assay (Charles River Laboratories, Charleston, USA) and level was below 0.16 EU/ml final concentration.

3.2.1.2. Plasma preparation

Plasma samples from endemic normals and LF-infected individuals were collected using a ficoll gradient centrifugation method [200,253]. 10 ml of patient blood were poured into the ficoll containing tubes (Greiner, Frickenhausen, Germany) and centrifuged at 800g for 20 min at room temperature. Thereafter, plasma samples were removed from the upper phase of the gradient, stored at -20°C in 1.8 ml cryo tubes (Nunc, Roskilde, Denmark) and then transferred to liquid nitrogen until used. For NEC individuals, plasma samples were isolated employing the same method with the following modifications being added: 35ml of PBS diluted blood was added to a 50ml Falcon tube containing 15ml ficoll.

3.2.1.3. Immunoglobulin isotyping

To analyze the immunoglobulin isotypes composition in IgG/IgG4 positive and negative fractions and in the plasma of EN and LF patients, ProcartaPlex Human Antibody Isotyping Panels (eBioscience, Vienna, Austria) were used according to manufacturer's instructions. Briefly, antibody coated magnetic bead mixtures were incubated with 25 µl of assay buffer, kit standards or diluted plasma samples (1:20000) in a ProcartaPlex 96-wells plates at room temperature for 1 hour. 25 µl of detection antibodies mixture was then added and the plates were incubated on an orbital shaker (Stuart, Staffordshire, UK) at 500 rpm for 30 min. After that, each well was incubated with 50 µl of diluted Streptavidin-Phycoerythrin for 30 min. All incubations were performed at room temperature in the dark (plate covered with black microplate lid) and the plates washed using a hand-held magnetic plate washer. Afterward, samples were suspended in 120 µl reading buffer. Data were acquired using a MAGPIX Luminex system (Luminex Cooperation) and analyzed with ProcartaPlex Analyst software 1.0.

3.2.1.4. Isolation of granulocytes

Granulocytes used in this study were purified from buffy coats of healthy European donors provided by the Institute for Experimental Haematology and Transfusion Medicine, University Clinic Bonn, Germany. Granulocytes were isolated using Ficoll-Hypaque (Pancoll, PAN Biotech, Aidenbach Germany) method. The density gradient was performed according to the manufacturer's instructions. Briefly: 15 mL heparinized venous blood samples were diluted with an equal volume of cold phosphate-buffered saline (PBS) in a 50 mL conical centrifuge tube, layered over 12 mL Ficoll, and centrifuged at 900g for 30 min at 4°C in a swinging bucket centrifuge (Thermo Scientific, Germany) with brake off. The opaque layer below the Ficoll/plasma interface containing granulocytes was transferred to another tube.

Thereafter, red blood cells were lysed by 10 min incubation at room temperature in 1x red blood cell lysis solution (Miltenyi Biotech, Bergisch Gladbach, Germany). Granulocytes were then centrifuged at 200g for 8 min at 4°C to remove contaminating red blood cells. Cell pellets were washed twice at 200g for 8 min in RPMI 1640 (Life Technologies, NY, USA) containing L-glutamine (292.3 µg/ml), gentamycin, and penicillin/streptomycin (50 µg/ml) (PAA, Linz, Austria). Supernatants were discarded, and the purity of isolated granulocytes was assessed by flow cytometry. The purity was routinely ≥ 96%. Following isolation, granulocytes were cultured in presence of plasma, IgG or IgG4 samples from endemic normal, NEC and LF-infected patients in a granulocyte suppression assay.

3.2.1.5. *In vitro* culture and granulocyte suppression and degranulation assays

For the assessment of modulation of granulocyte activity, isolated granulocytes were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 2 x 10^5 cells/well were plated and pre-incubated with 40 ng/ml of natural human IgE antibody (Abcam, Cambridge, UK) for 30 min at 37°C/5% CO_2 as previously described [254,255]. Then the cells were incubated at 37°C/5% CO_2 for 10 min in the presence of 2 ng/ml rhIL-3 (Miltenyi Biotech, Bergisch Gladbach, Germany) [256,257] and stimulated with 25 ng/ml anti-IgE mAb (Clone BE5) (Abnova, Taipei, Taiwan) and 10 µg/ml *Bm*Ag. Thereafter, granulocytes were incubated for 18 hours at 37°C / 5% CO_2 either alone or in the presence of plasma samples (5% v/v), 5 µg/ml IgG fractions or 2,5 µg/ml IgG4 antibodies purified from the plasma of EN, Mf+, Mf- and CP groups (sections 3.2.1.8 and 3.2.1.9). Supernatants were collected to assess granulocyte degranulation and cells were washed for flow cytometric analysis of granulocyte activation (section 3.2.1.6). To investigate the intracellular signaling pathways induced by IgG4 molecules, granulocytes were cultured at 37°C/5% CO_2 for 2 hours in presence of IgG4 from different groups by a modification of a previously described

method [258]. Then the phosphorylation or not of the kinases Syk, Src and SHIP1 was assessed using western blot (section 3.2.2.4) and phospho-flow cytometry (section 3.2.2.5).

3.2.1.6. Flow cytometry analyses

To assess granulocyte activation, cells were harvested from cultures and washed with FACS buffer (PBS / 2 % FCS) at 1300 rpm for 8 min. $2x10^5$ cells were resuspended in 100 µl of FACS buffer and blocked with 1µl of FC- block (Affymetrix eBioscience, San Diego, CA, USA) for 15 min. Then cells were incubated for 30 min at 4°C with $5µg/1x10^5$ cells either anti-human CD66b-FITC (clone: G10F5) and a mixture of 1) CD63-PE (clone: H5C6) and HLADR-FITC (clone: LN3) for granulocytes characterization or 2) anti-human CD63-PE-Cy7 (clone: H5C6), HLADR-APC (clone: LN3), CD15-PE (clone: HI98) and CD16-FITC (clone: CB16) for granulocyte neutrophils or 3) anti-human CD63-PE-Cy7 (clone: H5C6), HLADR-APC (clone: LN3), CD11b-PE (clone: ICRF44) and Siglec8-FITC (clone: 7C9) for granulocyte eosinophils or 4) anti-human CD63-PE-Cy7 (clone: H5C6), HLADR-APC (clone: LN3), CD203c-PE (clone: NP4D6) and CD123-FITC (clone: 6H6) for granulocyte basophils. Cells were then washed two times with FACS buffer and fixed in 4% PFA. To correct spectral overlaps, fluorescence compensation was done using UltraComp ebeads (Affymetrix eBioscience). The Data were acquired and analyzed using a FACS Canto flow cytometer and the BD-FACS-Diva analysis software (BD Biosciences). For gating strategy, cells were first gated for their size and relative granularity (FSC/SSC). Each cell population was then identified based on CD66b positivity for granulocyte characterization (Figure 7), CD15+/CD16+ for granulocyte neutrophils, CD11b+/Siglec8+ for granulocyte eosinophils and CD203c+/CD123+ for granulocyte basophils [259-262]. These positive and double positive populations are further analyzed for their activation characterized by CD63+/HLADR- expression.

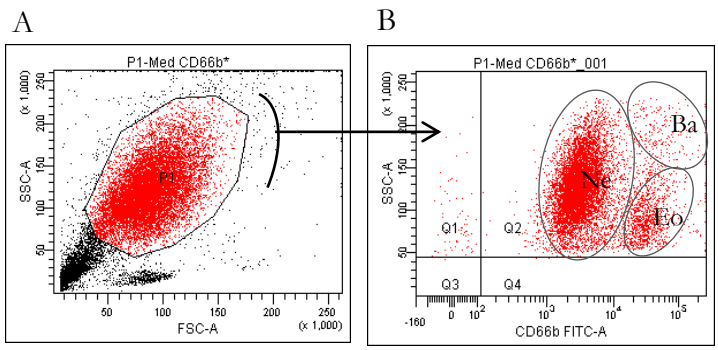

Figure 7: **Granulocyte characterization.** Freshly isolated granulocytes from healthy blood spenders were first gated for their size and relative granularity (FSC/SSC) (A) and then further analyzed for the expression of CD66b distinguishing neutrophils (Ne) from basophils (Ba) and Eosinophils (Eo) (B).

3.2.1.7. Assessment of granulocyte degranulation by ELISA

To analyze cell granule content release, granulocyte culture supernatants were collected and the levels of histamine, eosinophil cationic protein (ECP) and neutrophil elastase (NE) were analyzed using ELISA kits respectively from Abnova (Taipei, Taiwan), Abbexa (Cambridge, UK) and eBioscience (Vienna, Austria) according to the manufacturer's recommendations.

3.2.1.7.1. Histamine ELISA

Granulocyte culture supernatants and standards were first acylated by reacting 50 µl of samples, 25 µl of standards or control with 25 µl of acylation reagent and 25 µl of acylation buffer supplied in the test kit for 45 min. 25 µl aliquots of acylated standards, controls, and samples were pipetted into wells of the antibody-coated microplate provided with the kit. Then the wells received 100 µl of histamine antiserum, and the mixture was allowed to incubate for 3 hours at room temperature. The plates were then washed with the provided washing buffer to remove unbound materials. After that, the bound antibodies were detected using 100µl of anti-rabbit IgG-peroxidase conjugate using TMB as a substrate. The color was allowed to develop for 20 min at room temperature in the dark. The reaction was stopped, and the resulting OD values were measured at 450 nm. The histamine concentration, inversely

proportional to the OD was calculated using the SoftMax Pro Data Acquisition and Analysis Software.

3.2.1.7.2. Eosinophil cationic protein (ECP) ELISA

50 µl of prepared standards and culture supernatants were added to a microtiter plate wells pre-coated with a monoclonal antibody specific for ECP. After 1 hour incubation at room temperature, the wells were washed and incubated for 1 hour with 50 µl HRP-conjugated anti-ECP polyclonal antibodies. Thereafter the wells were thoroughly washed (5 times) to remove all unbound components. 50 µl TMB substrate solution was added to each well. The plate was gently shaken and after 15 min incubation, the enzyme-substrate reaction is terminated by addition of an acidic stop solution. The intensity of color developed is proportional to the concentration of ECP present in the sample, measured at 450 nm using the SpectraMAX ELISA reader (Molecular Devices, Sunyvale, USA). Data were analysed with SoftMax Pro 5.4.1 software.

3.2.1.7.3. Neutrophil elastase ELISA

Microtiter plates, pre-coated with an anti-human NE coating antibody, were incubated with 100 µl of prepared standards and prediluted supernatants (1:100) for 1 hour at room temperature on a microplate shaker (Grant Instruments, Cambridgeshire, UK) set at 400 rpm. Wells were washed delicately 4 times with 400 µl wash buffer provided in the kit and incubated with 150 µl HRP-conjugated detection antibody for 1 hour on the shaker. After thorough wash of the wells, 200 µl of TMB substrate solution are added for 20 min followed by 50 µl stop solution. The concentration of NE in each sample was determined at 450 nm.

3.2.1.8. Total IgG purification from plasma by affinity chromatography

Total IgG was isolated from the plasma of NEC, EN, Mf+, Mf- and CP using prepacked HiTrap™ Protein G columns (GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions. 100 µl of plasma samples were diluted with 1400 µl binding buffer (PBS, pH 7), provided in the kit, and passed through a pre-equilibrated protein G-Sepharose column (GE Healthcare, Freiburg, Germany) with a linear flow rate of 1 mL/min. Since Protein G binds to all human IgG subclasses, non-IgG plasma components were washed out from the column with PBS and collected as IgG negative fractions. Bound IgG was eluted in 1 ml fractions using IgG Elution Buffer (0.2 M Glycine/HCl pH 3.0) and immediately neutralized with 1:10 volume of saturated Tris-HCl (pH 9.0). The protein concentration of IgG fractions was then assessed at 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and the purity evaluated using western blot and Luminex assay (section 3.2.1.3).

3.2.1.9. IgG4 purification from IgG fractions by affinity chromatography

IgG4 antibodies were purified from IgG-enriched fractions using the CaptureSelect Human IgG4 affinity matrix (Life Technologies, Paisley, UK), containing an antibody fragment recognizing human IgG4, according to the manufacturer's instructions. Briefly, CaptureSelect affinity matrix was carefully packed and equilibrated in 10 ml affinity chromatography column with PBS (pH 7.3). Diluted IgG-enriched fractions (1:1 volume PBS) were loaded onto the column and the linear flow rate was 150 cm/hour. After washing unbound IgG4 negative components with PBS, the column was eluted with 0.1 M Glycine (pH 3.0) and the fractions were immediately neutralized with Tris-HCl (pH 9.0). IgG4 fractions were collected and the purity of fractions assessed by determining the level of IgG subclasses, IgA, IgE and IgM antibody by Luminex assay and western blot.

3.2.1.10. Western blot analysis of IgG and IgG4 fractions

The purity of eluted IgG and IgG4 fractions was analyzed by western blot. Samples were mixed with 2X reducing SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% fresh added β-mercaptoethanol) to a final dilution of 1X and heated at 95°C for 5 min. An equal quantity (2.5 µg) of the purified proteins and controls were loaded onto separate lanes of a polyacrylamide gel (4-12%) and resolved by SDS-PAGE (150 v; 45-60 min). The resolved proteins were transferred onto nitrocellulose membranes (GE Healthcare, Freiburg, Germany) using a Bio-Rad Trans-Blot Turbo Transfer system (Bio-Rad, Germany). The membranes were then blocked with gelatin blocking buffer (3% gelatin in Tris Buffered Saline (TBS)) (Bio-Rad, Germany) for 1 hour prior incubation with the primary antibody (polyclonal mouse anti-human IgG (H+L) or mouse anti-human IgG4 (1:1000 dilution)) (Thermo Fisher Scientific, Rockford, USA) for 1.5 hours at room temperature. The nitrocellulose membranes were then washed with TBS/0.05% Tween 20 before incubation for 1 hour with alkaline phosphatase-conjugated goat anti-mouse IgG (1:300 dilution) (Bio-Rad Laboratories, USA). Immune complexes were finally detected with NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Bio-Rad Laboratories, USA) and IgG characteristic bands analyzed.

3.2.2. Mechanisms of IgG4-mediated granulocyte inhibition in LF

3.2.2.1. Cytospin and immunofluorescence analysis of IgG4 binding on granulocytes

For the investigation of the Fc-receptors associated with IgG4-mediated granulocyte suppression, Granulocytes were cultured as previously described and surface FcγRs blocked or not. In the case where the receptors are blocked, blocking antibodies (10 µg/10^6 cells) against human FcγRI (clone: 10.1), FcγRII (clone: FUN-2) and FcγRIII (clone: 3G8) (all from Biolegend, San Diego, CA, USA) were added alone or in combination ((FcγRI/ FcγRII),

(FcγRI/FcγRIII), (FcγRII/FcγRIII)) for 1 hour at 37°C/5% CO_2 before the incubation with purified IgG4 antibodies. Control cells were incubated with IgG4 antibodies from all individual groups in absence of blocking antibodies. Then 2 x 10^5 cells were harvested and washed with PBS and 100 µl of diluted cells were aliquoted into cytospin funnels and spun at 4°C, 500g for 5 min onto glass slides (Engelbrecht, Edermünde, Germany) in a Hettich Cytospin centrifuge (Hettich, Tuttlingen, Germany). The slides were dried and the cells immediately fixed in 4% PFA for 15 min and washed twice with cold PBS. After that, the slides were blocked with PBS / 1% BSA for 30 min followed by incubation with the primary antibody (mouse anti-human monoclonal IgG4) (Thermo Fisher Scientific, Rockford, USA) for 1 hour. After washing 3 times, the slides were incubated with the Alexa Fluor 488 coupled secondary antibody (goat anti-mouse polyclonal IgG antibody) (Thermo Fisher Scientific, Rockford, USA) for 1 hour at room temperature in a humidifying chamber. Granulocytes were washed 3 times again and nuclear DNA was labeled with 0.25 µg/ml DAPI (Thermo Fisher Scientific, Rockford, USA) in PBS for 5 min. Cells were then washed once and mounted in VECTASHIELD-Antifade mounting medium (Vector Laboratories, CA, USA) and 6 fields were randomly chosen on the slides and the green fluorescence intensity, characterizing IgG4 binding on granulocytes, were measured using a Zeiss LM-Set Axiocam MRm microscope (Carl Zeiss, Thornwood, NY, USA) and data analyzed with Zen 2 software 1.0 (blue edition).

3.2.2.2. Protein extraction

To investigate the intracellular mechanisms induced by IgG4 binding on granulocytes by western blot, total protein was first extracted from granulocytes. Cultured cells were pelleted by centrifugation at 2500*g* for 10 minutes at 4°C. Cells were thereafter washed twice with cold PBS and incubated with a mix of a mammalian protein extraction buffer (100 µl per 1 x 10^6 cells) and a cocktail of protease and phosphatase inhibitors (the protease inhibitors target

aminopeptidases, cysteine and serine proteases and the phosphatase inhibitors target serine/threonine and protein tyrosine phosphatases) (all from Thermo Fisher Scientific, Rockford, USA). The lysate was collected, the cell debris centrifuged at 14000*g* for 15 min at 4°C and supernatant transferred to a new tube. Protein concentration of the lysate was determined by Bradford protein assay and lysate samples frozen at -80°C or used for immediate western blotting analysis of the phosphorylation of Src, syk and SHIP1 kinases (section 3.2.2.4).

3.2.2.3. Bradford protein assay

To determine the protein concentration of lysate, a Bradford protein assay was performed as described by the manufacturer's protocol. In brief, serial dilutions of bovine serum albumin (BSA) was performed and used as standards against the lysate samples. Another serial dilution of the samples was done in PBS. 300µl per well of Coomassie blue G-250 (Cytoscelecton, Denver, USA) reagent was distributed in duplicate in an ELISA plate and 3µl of diluted samples and standard were added accordingly. The protein concentration in lysate was determined at 595 nm using a SpectraMAX 190 microplate reader (Molecular Devices, California, USA).

3.2.2.4. Analysis of IgG4-mediated intracellular signaling pathways by western blot

To investigate IgG4-induced proteins and phospho-proteins either of the activation signaling pathways, characterized in our settings by Src, Syk kinases and the related phosphorylated forms (pSrc, pSyk), or the inhibition signaling pathways, characterized by SHIP1 and pSHIP1 kinases, granulocytes were cultured as previously described in presence or absence of IgG4 molecules from EN, Mf+, Mf- and CP groups (section 3.2.1.5). Total protein was extracted from the cells and protein concentration determined by Bradford assay. The phosphorylation or not of Src, Syk and SHIP1 kinases was analyzed by western blot as described in section

3.2.1.10 with the following modifications: protein lysates were separated by SDS-PAGE using 4-12% polyacrylamide gel for Src, Syk, β-actin and 4-10% polyacrylamide gel for SHIP1 and 25 μg of proteins were loaded per lane. β-actin is used as control. Membranes were incubated with rabbit anti-human antibodies specific for the unphosphorylated and phosphorylated forms of Src, Syk, β-actin and SHIP-1 (1:1000) (all from Cell Signaling Technology, Beverly, USA). Rabbit antibodies were detected with goat anti-rabbit alkaline phosphatase-linked antibody (1:2000) (Cell Signaling Technology, Beverly, USA). In this experimental setting, we were not able to detect the presence of SHIP1 as well as pSHIP1 proteins. We then used a more sensitive method, the phospho-flow cytometry, to assess the phosphorylation of SHIP1.

3.2.2.5. Phospho-flow cytometry

Granulocytes were cultured in presence or absence of IgG4 molecules from different groups. Cells were harvested from cultures, washed with FACS buffer and blocked with FC- block (Affymetrix eBioscience, San Diego, CA, USA). The intracellular expression of SHIP1 and phospho-SHIP1 were measured by flow cytometry using BD Phosflow (BD Biosciences) protocol for human whole blood sample. Cells were fixed in Phosflow Lyse/Fix Buffer (20:1 cell volume) (BD Bioscience, Heidelberg, Germany**)** for 10 min at 37 °C. After 10 min washing at 300g, cells were permeabilized with Phosflow Perm/Wash Buffer I (BD Bioscience, Heidelberg, Germany) for 10 min at room temperature. Then granulocytes were washed twice and incubated with rabbit anti-human phospho-SHIP antibody (1:300) (clone: 3635) (Stemcell Technologies, Köln, Germany) for 45 min followed by FITC-conjugated goat anti-rabbit secondary IgG antibody (1:300) (clone: 6717) (Cambridge, UK) for 45 min at 4°C in the dark. All these data were accessed by FACS Canto flow cytometer using BD-FACS-Diva analysis software (BD Biosciences).

3.2.3. Modulation of complement activation in LF

3.2.3.1. Complement C1q level determination in plasma

The level of complement first component C1q in subjects' plasma samples was determined using a specific ELISA kit from eBioscience (Vienna, Austria). Microtiter plates provided in the kit were pre-coated with an anti-human C1q antibody. After thorough washing of the wells, 100 µl of prediluted plasma samples (1:100) and prepared standards were added to their respective wells and plates were allowed to incubate for 2 hours at room temperature on a microplate shaker (Grant Instruments, Cambridgeshire, UK) set at 400 rpm. Wells were then washed 6 times and each well was filled with 100 µl of prepared biotin-conjugated detection antibody and incubated for 1 hour on microplate shaker. Thereafter the plates were washed and 100 µl of streptavidin-HRP were added to the wells for 1 hour with shaking in the dark. After a final washing step, wells were incubated with 100 µl of TMB substrate solution for 30 min and substrate reaction stopped with acidic stop solution. The absorbance of each microwell was read at 450 nm using the SpectraMAX ELISA reader (Molecular Devices, Sunyvale, USA) and the concentration of C1q in samples determined.

3.2.3.2. Determination of CIC-C1q levels

To analyze the quantity and interaction of Circulating Immune Complexes (CIC) from plasma from different groups with complement first component C1q, an enzyme immunoassay kit from Quidel Corporation (San Diego, CA, USA) was used according to manufacturer's instructions. Briefly, 100 µl reconstituted standards and diluted plasma samples (1:50) were added to the C1q-precoated and rehydrated microtiter wells and incubated for 1 hour at room temperature. After wells had been washed 5 times, 50 µl of horseradish peroxidase-conjugated goat anti-human IgG were dispensed in each well for 30 min to detect bound CIC. An additional wash procedure was performed followed by 30 min incubation with 100 µl of

substrate solution. The reaction was stopped with 50 µl of acidic solution. The absorbance of each well was measured at 405 nm and is proportional to the amount of CIC binding the solid-phase C1q. Results are expressed as heat aggregated human gamma globulin equivalents per mL (µg Eq/ml).

3.2.3.3. Immunoglobulins-C1q specific ELISA

To determine the binding capacity of immunoglobulin isotypes from plasma samples to C1q, high binding ELISA plates (Greiner Bio-One, Frickenhausen, Germany) were coated with 50 µl recombinant human complement C1q (Sigma-Aldrich, Saint Louis, Missouri, USA) at the concentration of 1µg/ml and incubated at 4°C overnight. The plates were then washed 5 times with PBS/0.05% Tween 20 and blocked with PBS/1% BSA for 1 hour at room temperature. The wash step was repeated and subsequently, plates were incubated overnight at 4°C with 50 µl/well of plasma samples at various dilutions ((1:1000 for IgG1-2 binding), (1:500 for IgG4, IgA, IgE) and (1:2000 for IgG3, IgM)). Wells were further washed and diluted biotin-conjugated anti-IgG1, IgE (1:1000); IgG2, IgG4, IgA (1:15000); IgG3, IgM (1:4000) (all from Sigma Aldrich, Saint Louis, Missouri, USA) were added, followed by incubation at room temperature for 2 hours. Finally, plates were again washed and afterwards incubated with 50 µl/well of Streptavidin-HRP for 45 min in the dark. After a final washing step, 50 µl/well TMB substrate solution were added to the plates and the reaction was stopped 15 min later with 25 µl/well 2N H2SO4 stop solution (Merck KGAA, Darmstadt, Germany). Optical density was measured at 450 nm using the SpectraMAX ELISA reader and the results were expressed as arbitrary units (AU) relative to a standard serum arbitrarily set at 5 AU.

Thereafter the C1q-binding capacity of immunoglobulins in plasma from different individuals to complement C1q in absence of IgG4 was investigated. Therefore, IgG4 antibodies were removed from the plasma samples by affinity chromatography as described in section 3.2.1.9

and the binding capacity of immunoglobulins to complement was assessed as described above.

3.2.4. Statistical analysis

To determine statistical differences between the different groups, the software PRISM 5.02 (GraphPad Software, Inc., La Jolla, USA) was used. Comparative analyzes among groups were conducted using either ANOVA or the Kruskal-Wallis test with a Dunn's nonparametric post-hoc test (> 2 groups). In case of two groups, Student's t test or Mann-Whitney U test was used. Significance was accepted when $p < 0.05$. *p < 0, 05, **p < 0, 01 and ***p < 0, 001.

4. RESULTS

4.1. Modulation of granulocyte activation and functions in LF

4.1.1. IgG4 is preferentially expressed in the plasma of Mf+ individuals

To define the initial antibody profile of EN, Mf+, Mf- and CP in plasma samples we used in this study, we compared the plasma levels of IgG1, IgG2, IgG3, IgG4, IgE, IgM and IgA in different groups using a Luminex-based immunoassay (Figure 8). We found that the IgG1 expressions observed in EN and CP were similarly high. In contrast, Mf+ and Mf- individuals presented relatively moderated IgG1 levels (Figure 8A). However, while the highest levels of IgG2 were detected in the plasma of CP individuals, plasmatic IgG2 in EN and Mf- were significantly lower compared to Mf+ and CP (Figure 8B). No significant differences could be observed in the expression of IgG3 between the 4 groups (Figure 8C). Interestingly, the expression of IgG4 was relatively low in EN, Mf- and CP but significantly elevated in Mf+ (Figure 8D). This contrasts with lower levels of IgE in those patients in comparison to Mf- and patients with chronic pathological manifestations (Figure 8E). In addition, only EN has displayed significant plasmatic IgA expression (Figure 8F), whereas no significant differences were seen in the expression of IgM (Figure 8G). Thus, increased IgG4 characterized the Mf+ group and the ratio IgE/IgG4 negatively correlate with the presence of worms. These results indicate that IgG4 and IgE are respectively associated with worm's establishment and disease manifestations whereas IgA and IgG1 are prominent in putative immunity.

As plasma from Mf+ patients contained high levels of anti-inflammatory IgG4 antibody and known the anti-inflammatory properties that characterize IgG4 antibodies, we hypothesized that plasma from Mf carriers, and specifically IgG4 molecules, would preferentially down-modulate granulocyte activation and degranulation. We then next investigated how crude

plasma of NEC, EN, Mf+, Mf- and CP modulate the function of IL-3/IgE/*Bm*Ag activated granulocytes.

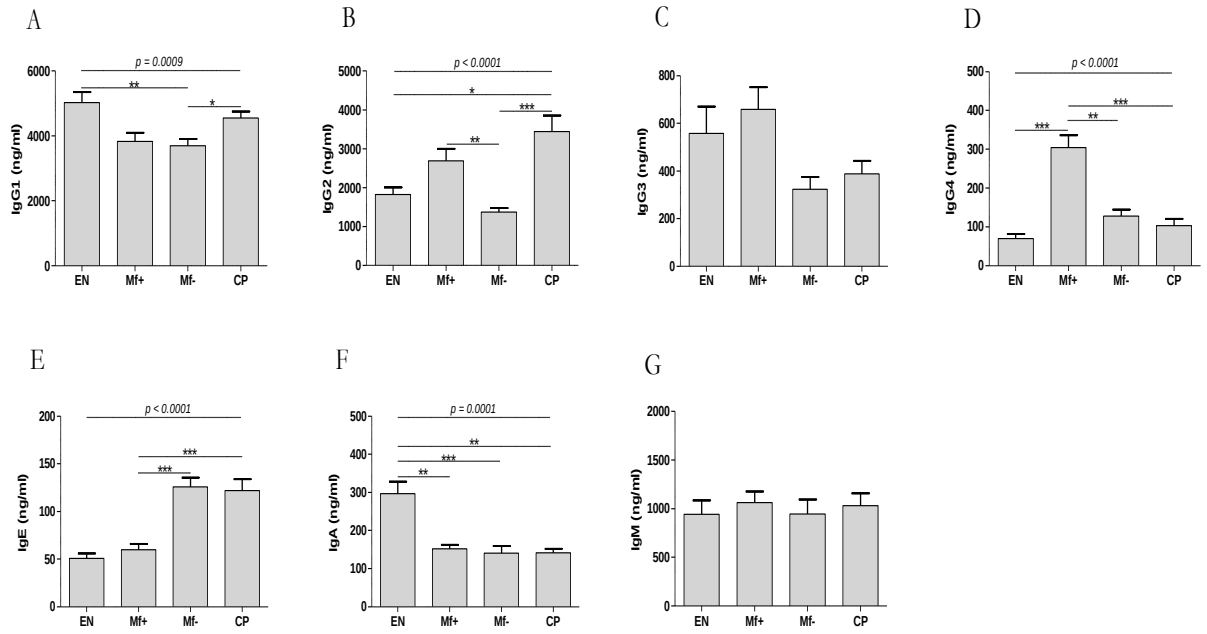

Figure 8: **Preferential expression of IgG4 in Mf+.**

10 µl of plasma samples from EN (n=14) and LF infected Mf+ (n=14), Mf-(n=14) and CP (n=14) patients were diluted and analyzed for the expression of IgG1-4 (A-D), IgE (E), IgA (F) and IgM (G) using Luminex-based immunoassay. Bars depict the plasmatic antibody concentration as mean ± SEM. Asterisks show statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups indicated by the lines. *P < 0.05; **P < 0.01; ***P < 0.001.

4.1.2. Plasma from EN and Mf+ but not those of Mf- and CP impaired granulocyte activation

To investigate the effect of plasma from different groups on granulocyte activation, we first isolated granulocytes from healthy blood spenders and cells were plotted to determine their positions by forward and side scatter parameters and gated as previously described [262]. Then, gated granulocytes were further characterized for CD66b expression. The analysis of the expression of granulocyte specific marker CD66b on the gated cells showed three distinct populations (Figure 7, section 3.2.1.6): a large population of granulocytes with medium

expression of CD66b that we referred as neutrophils [259,261]; two populations with high CD66b expression, one with high granularity, characterized as basophils [260] and the other less granulated, the eosinophils [261]. After granulocyte characterization, stimulated cells were analyzed for their activation in presence of individuals' plasma by monitoring the expression levels of CD63/HLADR (Figure 9). CD63 characterized activated granulocytes and HLADR is a negative control marker for granulocytes [117,119,120,263,264]. We observed that, compared to the control (Figure 9A), whereas plasma from NEC has no effect on granulocytes (Figure 9B), those from Mf- and patients with chronic pathological manifestations not only failed to suppress granulocytes but also slightly enhanced cell activation (Figure 9E, F). Importantly, plasma

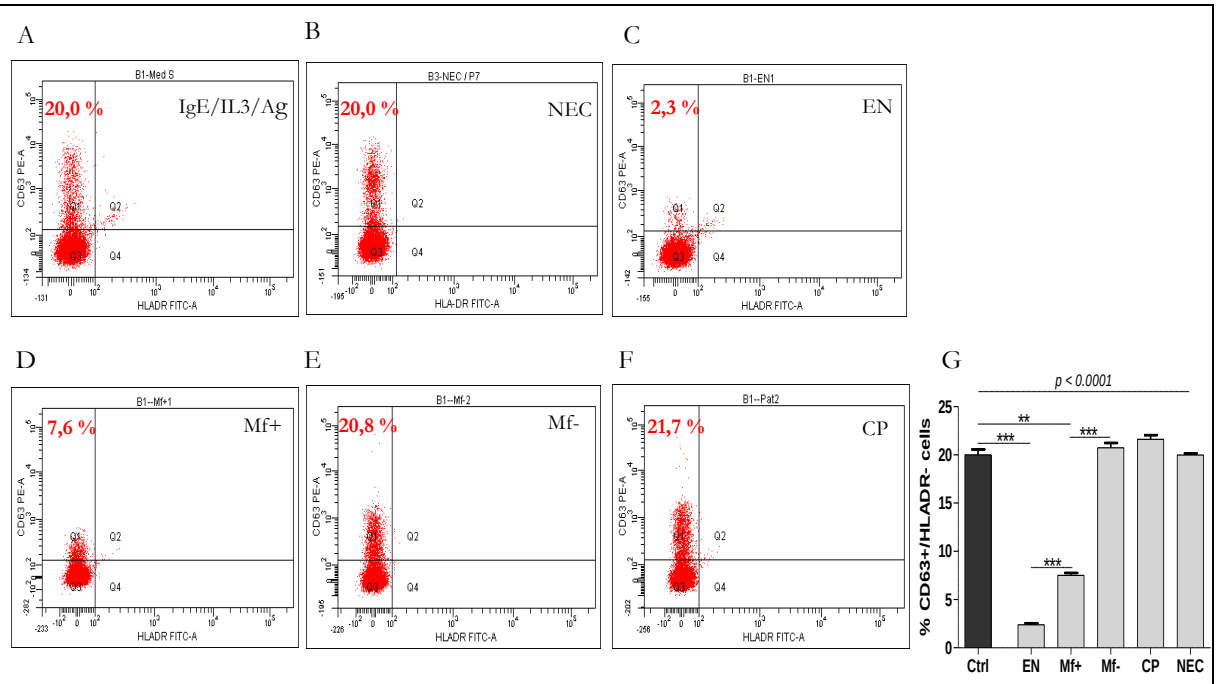

Figure 9: **Plasma from EN and Mf+ patients suppress granulocytes activation**. 2 x 10^5 cells/well of freshly isolated granulocytes from healthy blood spenders (n=9) were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml) and Brugia antigen extracts (10 µg/ml) as control (A) (dark bars) and then cultured in presence of 5% (v/v) of plasma of either non-endemic controls (NEC) (B), endemic normal (EN) (C), microfilaria positive (Mf+) (D), microfilaria negative individuals (Mf-) (E) or plasma of chronic pathology patients (CP) (F) (grey bars). Cells were then stained for CD63 and HLADR antigens expression. The percentage of activated granulocytes (CD63+/HLADR- cells) was determined after 18 hours of incubation. Bars represent means ± SEM of the percentage of CD63+/HLADR- cells in 3 independent experiments. Asterisks show statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. **P < 0.01; ***P < 0.001.

from EN and Mf+ have significantly inhibited the activation of granulocytes as indicated by the lower percentages of CD63+HLADR- cells (Figure 9C, D, G) suggesting that a pool of components present in both plasma at different dose might be responsible for this activity. Interestingly, the plasma of EN presented a higher inhibitory potential on granulocytes activation when compared to those of Mf+. These results indicate that, in lymphatic filariasis, active factors in EN and Mf+ infected patients' plasma environment but not present in Mf- and CP patients impaired granulocytes activation. We, thereafter, analyzed the degranulation of the cells.

4.1.3. Release of histamine and elastase inhibited in presence of plasma from EN and Mf+ whereas only Mf+ reduced ECP release

Following granulocyte activation tests, we assessed granulocyte degranulation by analyzing the release of granule components (histamine, eosinophil cationic protein (ECP) and neutrophil elastase (NE)) in culture supernatants. In line with the activation data (section 4.1.2), plasma from both EN and Mf+ significantly suppressed the release of histamine and NE (Figure 10A, B) as diminished levels of these mediators are detected in supernatants. However, while the plasma of Mf+ individuals significantly prevented the release of ECP in granulocyte cultures, those of EN failed to suppress ECP release (Figure 10C). Again we could not detect an effect from Mf- and CP plasma samples. Moreover, CP plasma slightly enhanced granule contents release. Thus during lymphatic filariasis infection, plasma from EN and Mf+ individuals harbour components that negatively regulate granulocyte degranulation, especially neutrophils and basophils' degranulation but not eosinophils'.

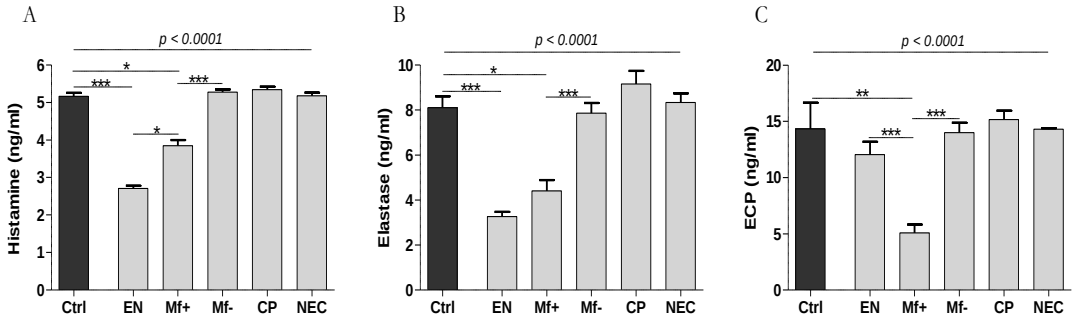

Figure 10: **Plasma from EN and Mf+ differently modulate granulocyte degranulation**. 2 x 10^5 cells/well of freshly isolated granulocytes from healthy blood spenders (n=9) were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml) and Brugia antigen extracts (10 µg/ml) as control (dark bars) and then cultured in presence of 5% (v/v) of plasma of either non-endemic controls (NEC), endemic normal (EN), microfilaria positive (Mf+), microfilaria negative individuals (Mf-) or plasma of chronic pathology patients (CP) (grey bars). After 18 hours incubation, histamine (A), neutrophil elastase (B) and eosinophil cationic protein (C) were measured in the culture supernatants. Bars represent means ± SEM of the concentration of histamine, elastase and ECP. Data are representative for 3 independent experiments. Asterisks show statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; **P < 0.01; ***P < 0.001.

4.1.4. Inhibition of granulocyte activation and degranulation originated from non-IgG factors in EN but from IgG and non-IgG factors in Mf+

To define the role of IgGs in the suppression of granulocytes by plasma of EN and Mf+, we depleted IgG antibodies from whole plasma per affinity chromatography using protein G matrix and tested the ability of IgG positive and negative fractions to modulate granulocyte functions. The purity of IgG positive and negative fractions was analyzed by western blot under reducing conditions (Figure 11A, B) and additionally the concentrations of IgG1-4, IgE, IgA and IgM in both fractions were determined by Luminex bead assay (Figure 11C, D). With IgG-enriched fractions from EN, Mf+ and Mf- (Figure 11A), we detected the three characteristic bands of IgG: the IgG heavy chain (50 kDa), the IgG light chain (25 kDa) and a third band (150 kDa) representing whole IgG molecule that have not been fully denatured. In the negative fractions, no IgG bands were detectable (Figure 11B). However, the IgG light chain from CP plasma appears unclear and fragmented in smaller bands. Consequently, in

immune context, this may lead to a different pattern of activity compared to that could be observed with other groups.

We further analyzed the purity of eluted fractions by Luminex assay by assessing the levels of IgG1-4, IgE, IgA and IgM in IgG positive (Figure 11C) and IgG negative (Figure 11D) fractions. As demonstrated in figures 11C and 11D, no or quite few contaminations of non-IgG antibodies in IgG eluates and IgG antibodies in non-IgG eluates were detected.

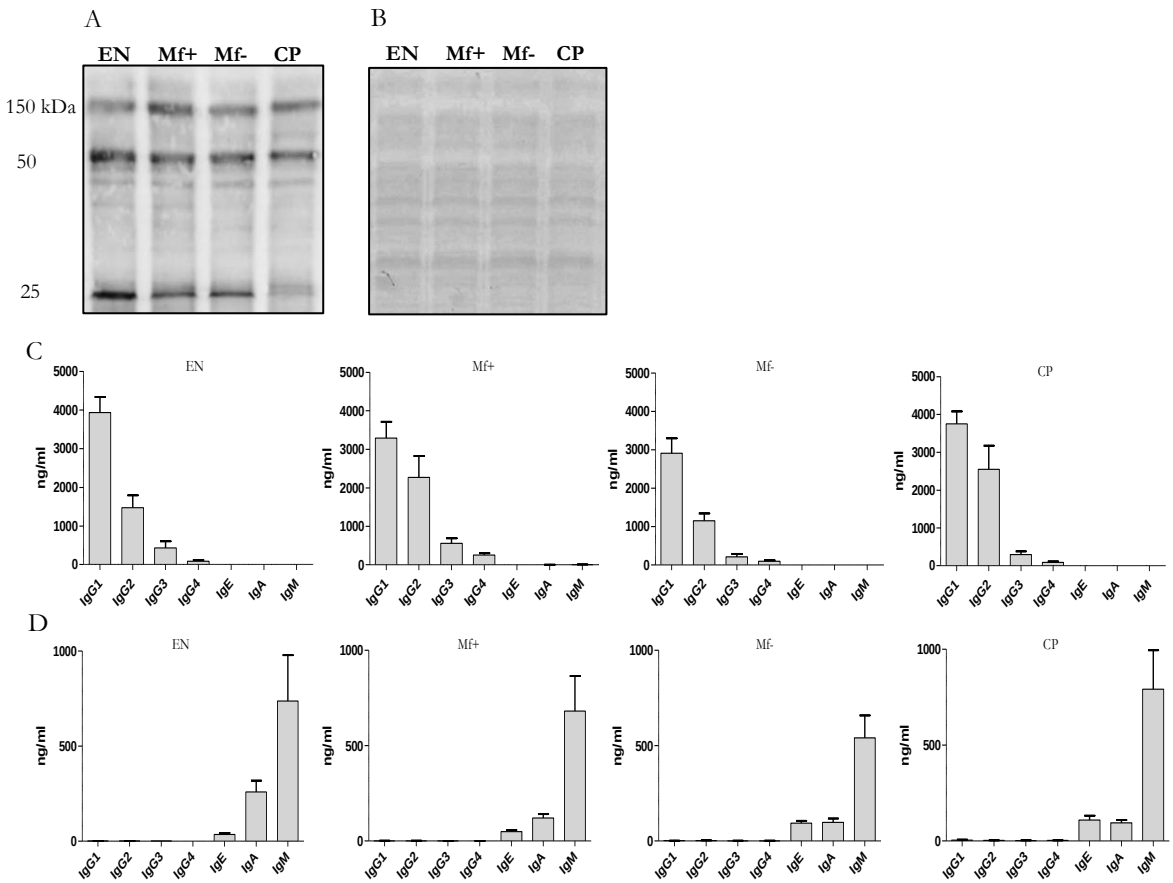

Figure 11: **Purity of IgG fractions.** IgG antibodies were depleted from the plasma of EN, Mf+, Mf- and CP using protein-G based affinity chromatography. Western blot analysis was performed on both eluates (A) and negative fractions (B) for IgG characteristic bands. Levels of IgG1-4, IgE, IgA and IgM in purified IgG positive (C) and negative (D) fractions were determined by Luminex. These results (A and B) are representative for 3 independent experiments. Bars indicate means ± SEM of the concentration of immunoglobulins in purified IgG fractions.

After purity control, we investigated whether and to which extend purified IgG antibodies from different groups could modulate granulocyte activation and degranulation (Figure 12). Interestingly, while IgG negative (IgG-) fractions of EN significantly suppressed granulocyte activation, IgG positive (IgG+) fractions had no effect (Figure 12A). More interestingly, both IgG+ and IgG- fractions from Mf+ significantly inhibited the activation of the cells (Figure 12B) whereas neither IgG+ nor IgG- fractions from Mf- and CP affected granulocytes activation (Figure 12C, D). Moreover, in Mf+, the IgG-related inhibition was significantly higher than that observed with negative fractions but not than the granulocyte inhibition potential observed with IgG- fractions from EN. These trends were also reflected after release

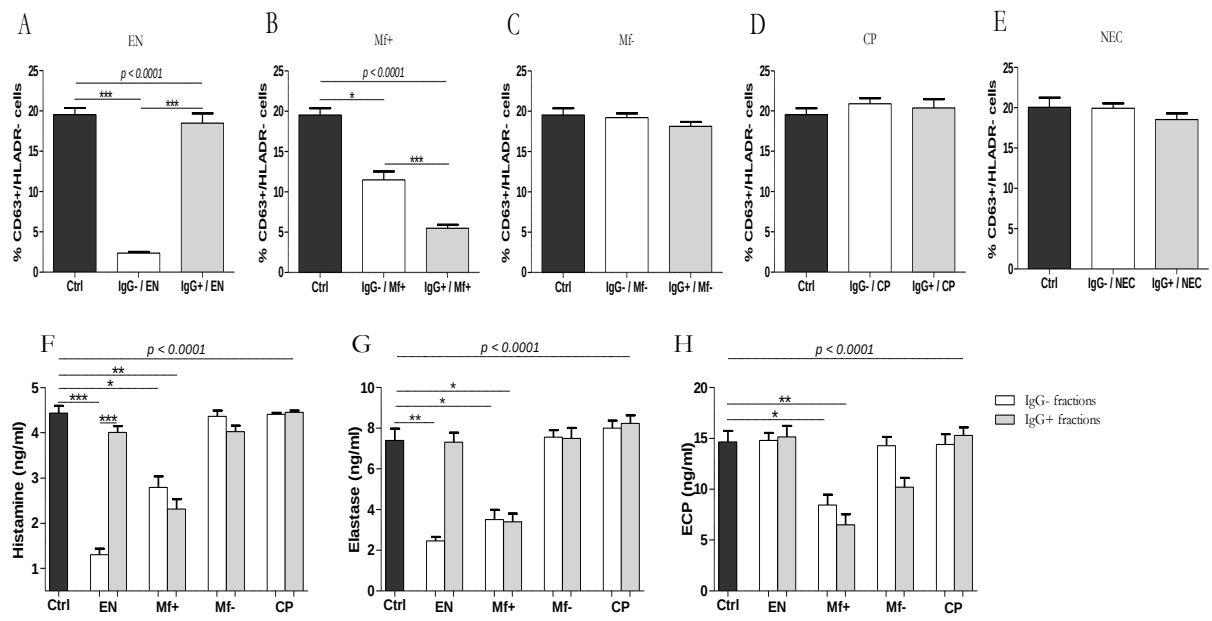

Figure 12: **Suppression of granulocytes activation and degranulation is IgG-independent in EN but IgG-dependent in Mf+.** 2 x 10^5 cells/well of freshly isolated granulocytes from healthy blood spenders (n=9) were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) alone (dark bars) or in presence of 5 µg/ml of IgG negative fractions (n=8) (light bars) from EN (A), Mf+ (B), Mf- (C), CP (D) and NEC (E) or the corresponding IgG positive fractions (grey bars). Thereafter cells were stained for CD63 and HLADR antigens expression. Activated granulocytes were characterized as CD63+/HLADR- cells. Bars represent means ± SEM of the percentage of activated granulocytes. The release of histamine (F), neutrophil elastase (G) and eosinophil cationic protein (H) in culture supernatants was assessed after 18 hours. Asterisks show statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; **P < 0.01; ***P < 0.001.

of histamine (Figure 12F) and neutrophil elastase (Figure 12G) as reduced release of both soluble mediators was measured in supernatants. Surprisingly both fractions from EN did not impair ECP release in comparison with histamine and elastase when IgG+ and IgG- fractions impaired ECP release for the same assay (Figure 12H). These data suggest that whereas total IgG from Mf+ individuals inhibited granulocyte activation, IgG-independent factors, absent in Mf+ or at lower dose, are involved in the suppression by plasma from EN.

4.1.5. IgG4 from EN, Mf+ and Mf- but not CP dampened granulocyte functions in a dose-dependent manner

We next investigated whether the modulation of granulocytes activation and degranulation by Mf+ IgG fractions is associated with the presence of the anti-inflammatory isotype IgG4. Highly pure fractions of IgG4 antibodies were prepared from IgG positive fractions using a specific affinity chromatography matrix containing an antibody fragment recognizing human IgG4 (Figure 13A). Then the purity of IgG4 eluates was validated by western blot and Luminex assay as described in section 4.1.4. The three characteristic bands of IgG4 were detected: the heavy chain, the light chain and the whole IgG4 molecule (Figure 13B). The analysis of immunoglobulin levels in IgG4+ fractions by Luminex showed no contamination of IgG4 fractions form EN, Mf- and CP whereas few minor and not significant levels of IgG1 and IgM were detected in IgG4 fractions from Mf+ (Figure 13C).

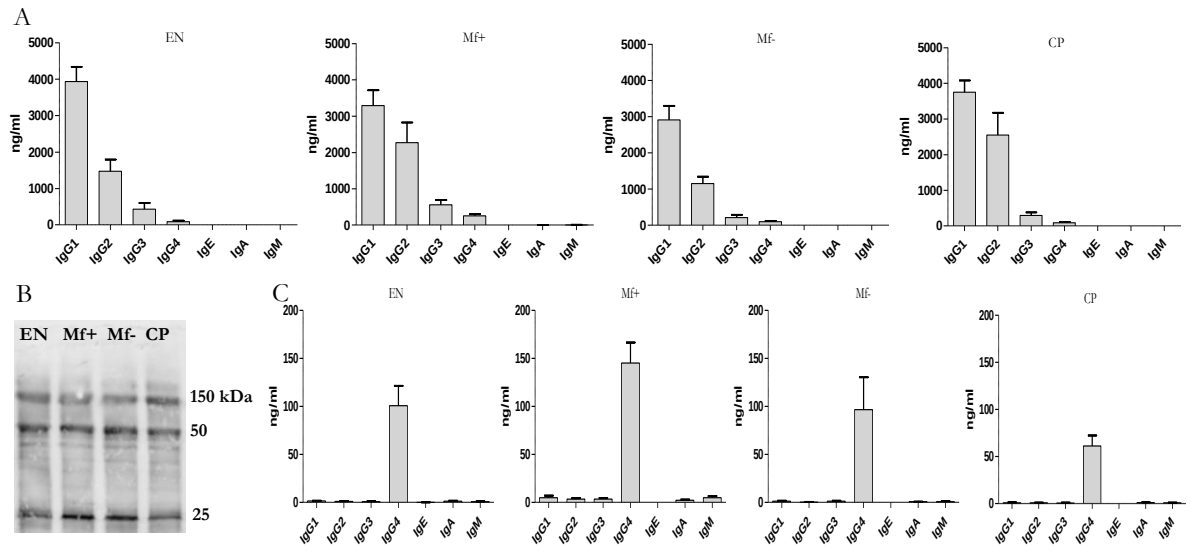

Figure 13: **Purity of IgG4 eluates.** IgG4 antibodies were depleted from the IgG+ fractions of EN, Mf+, Mf- and CP (A) using an IgG4 affinity chromatography matrix. Western blot analysis was performed on IgG4 eluates (B) for IgG characteristic bands and levels of IgG1-4, IgE, IgA and IgM in purified IgG4+ fractions from EN, Mf+, Mf- and CP (C) were determined by Luminex. The results (B) are representative for 3 independent experiments. Bars indicate means ± SEM of the concentration of immunoglobulins in IgG and IgG4+ fractions.

Thereafter the purified antibodies were tested on activated granulocytes. Strikingly, while IgG4 antibodies from EN, Mf+ and Mf- significantly inhibited granulocyte activation (Figure 14A-C), those from CP failed to suppress cell activation (Figure 14D) compared to the control. In addition, the suppressive effect was completely abrogated after IgG4 removal from IgG fractions (Figure 14A-C). As IgG4 from CP exhibited a slight but not significant inhibition potential, we next investigated whether the inhibition effects we observed were dose-dependent. Therefore, we conducted dose-dependent assays by testing increasing concentrations (1.25 µg/ml, 2.5 µg/ml and 5 µg/ml) of IgG4 antibodies from different groups. The results revealed that, whereas increasing concentrations of IgG4 from EN, Mf+ and Mf- proportionally reduced the percentage of activated cells (CD63+/HLADR-) in a dose-dependent manner, no dose effect was seen when IgG4 from pathology patients were used (Figure 14E), suggesting a functional difference between IgG4 from CP and those from EN, Mf+ and Mf-. Moreover, at the highest concentration, no difference in granulocyte inhibition

could be observed with IgG4 from EN, Mf+ and Mf-. Consistent with the granulocyte activation data, we detected lower levels of histamine and elastase in supernatants of granulocyte cultures treated with IgG4 antibodies from EN, Mf+, and Mf- compared to those treated with IgG4 from CP (Figure 15A-B). However, no significant reduction in the release of ECP was observed after incubation with IgG4 from EN and Mf- (Figure 15C). Again the removal of IgG4 abrogated the suppressive effect of IgG-enriched fractions. Thus, IgG4 antibodies produced in EN and Mf- individuals during lymphatic filariasis infection, affect degranulation of neutrophils and basophils but not eosinophils' whereas in Mf+, the degranulation of the three cell types is impaired.

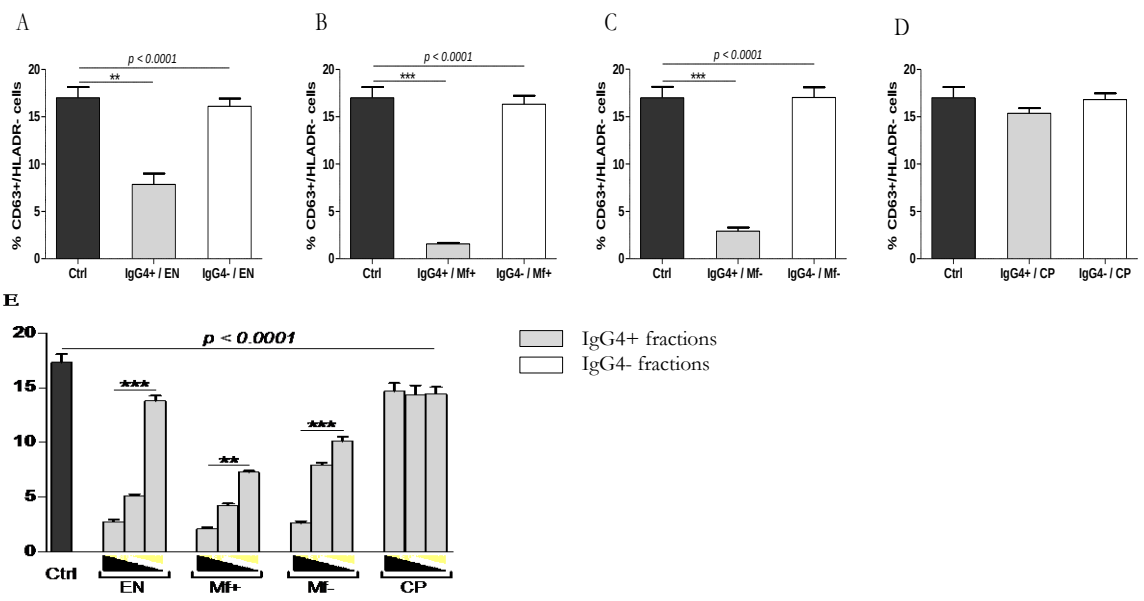

Figure 14: **Depletion of IgG4 abrogates the suppressive capacity of IgG positive fractions from LF infected individuals.** 2 x 10^5 cells/well of freshly isolated granulocytes from healthy blood spenders (n=9) were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) alone (dark bars) or in presence of 2.5 µg/ml of IgG4 negative (light bars) or positive (grey bars) fractions (n=8) from EN (A), Mf+ (B), Mf- (C), CP (D) and increasing concentrations (1.25 µg/ml, 2.5 µg/ml, 5 µg/ml) of IgG4 positive fractions from different groups (E). Activated granulocytes were characterized as CD63+/HLADR- cells. Bars represent means ± SEM of the percentage of CD63+/HLADR- cells. Asterisks show statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. **P < 0.01; ***P < 0.001.

Altogether, these results indicate that the suppressive effect of IgG fractions stems from IgG4 molecules and not from other IgG antibodies.

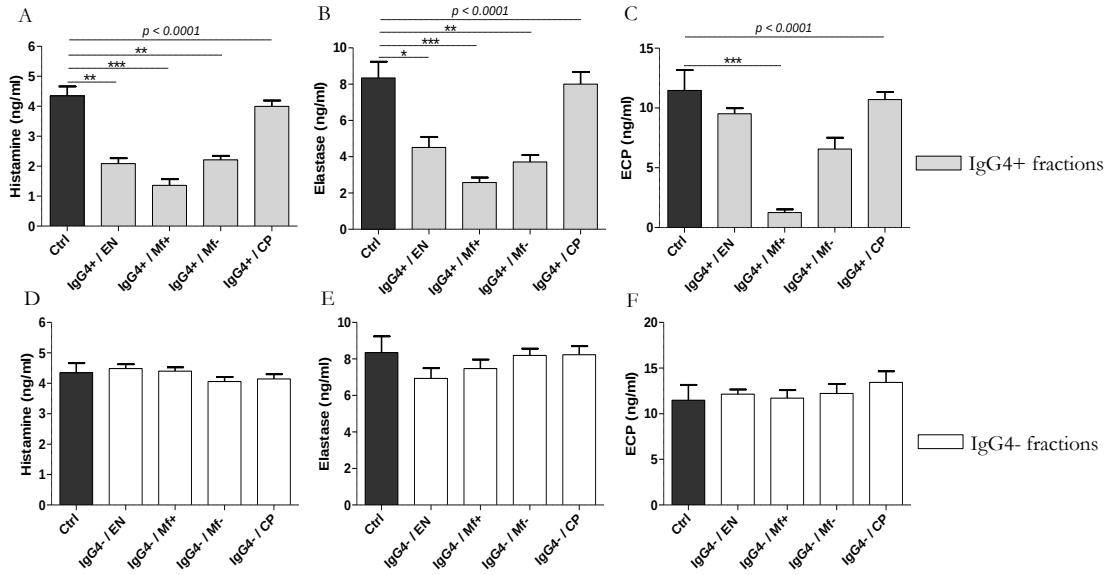

Figure 15: **Inhibition of degranulation in IgG+ fractions stems from IgG4.** 2 x 10^5 cells/well of freshly isolated granulocytes from healthy blood spenders (n=9) were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) alone (dark bars) or in presence of 2.5 µg/ml of IgG4 negative fractions (n=8) (light bars) from EN, Mf+, Mf-, CP or the corresponding IgG4 positive fractions (grey bars). The release of histamine (A and D), neutrophil elastase (B and E) and eosinophil cationic protein (C and F) in supernatants was assessed after 18 hours. Bars represent means ± SEM of the concentration of histamine, elastase and ECP. Asterisks show statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; **P < 0.01; ***P < 0.001

4.1.6. IgG4 reduced granulocyte neutrophils and basophils activation but not eosinophils activation

We have shown that IgG4 from EN, Mf+ and Mf- individuals impaired granulocyte activation and degranulation. Granulocytes are classically composed of neutrophils, eosinophils and basophils. These cells have distinct functions during infections with filarial worms. In order to distinguish between which critical functions of granulocyte are impaired, we next investigated which granulocyte subtypes are affected by the IgG4-mediated suppression. For this purpose, granulocytes were cultured in the presence of purified IgG4 antibodies from different groups and neutrophil, eosinophil and basophil populations were

gated from granulocytes as described in section 3.2.1.6 and the activation of each granulocyte subtype population was measured by flow cytometry. As shown in figures 16C, D, F and H, incubation of granulocytes with IgG4 from Mf- and EN individuals reduced the percentage of activated neutrophil cells (CD15+/CD16+/CD63+/HLADR-) from 27,5 % to 13,6 %. This reduction is even accentuated following incubation with IgG4 from Mf+ patients (5,5 % neutrophils activation) (Figure 16E, H) but no significant effect on neutrophils was detected in presence of IgG4 from CP (Figure 16G-H).

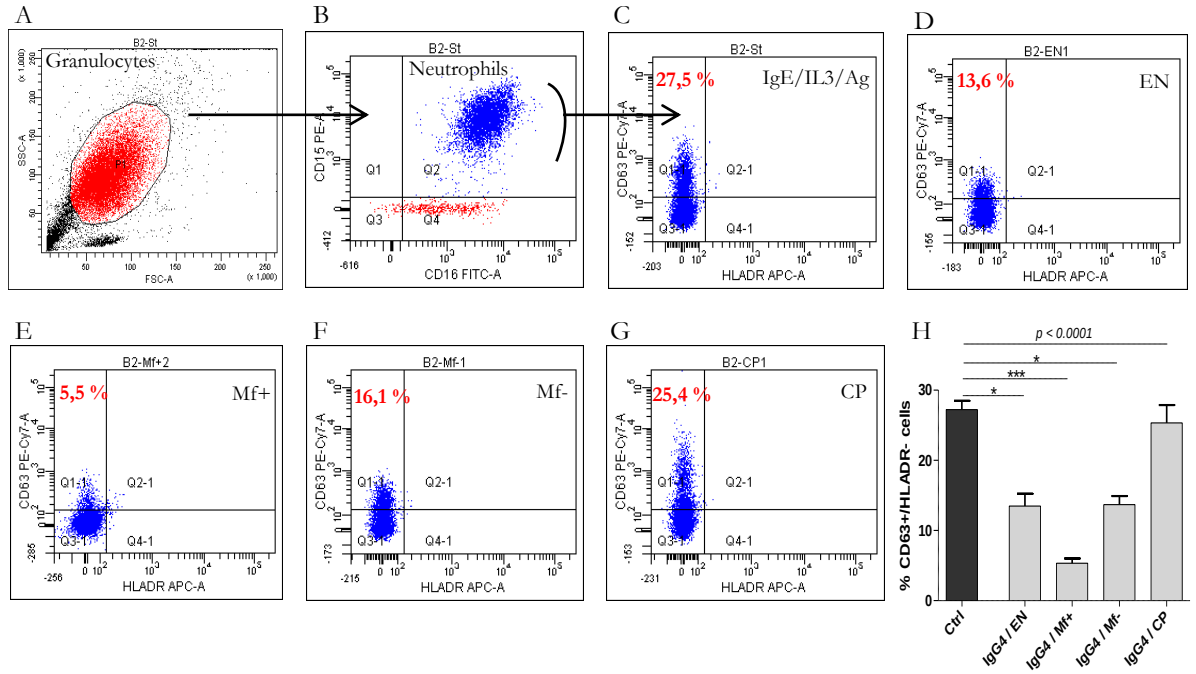

Figure 16: **IgG4 from EN, Mf+, Mf- but not CP reduced neutrophil activation**. Granulocytes were isolated from healthy blood spenders (n=9) and 2 x 10^5 cells/well were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) alone (C) (dark bars) or in presence of 2.5 µg/ml of IgG4 antibodies (n=8) (grey bars) from EN (D), Mf+ (E), Mf- (F) and CP (G) for 18 hours. Neutrophils population was gated as CD15+/CD16+ cells (B) from granulocytes population (A) and further analyzed for activation characterized by CD63+/HLADR- expression (C-H). Bars represent means ± SEM of the percentage of activated neutrophils. Asterisks indicate statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; ***P < 0.001.

Similar results were obtained as we looked on basophils' activation in presence of IgG4 from different groups (Figure 17). Indeed the activation of basophils induced by stimulation with

IL-3 and IgE (Figure 17C) was strongly reduced after incubation with IgG4 from EN (Figure 17D, H), Mf+ (Figure 17E, H) and Mf- (Figure 17F, H). Interestingly when compared with neutrophils, basophils presented an impressive activation after stimulation with IL-3 and IgE. This intense activation is almost totally inhibited by IgG4 from Mf+ patients. Still we could detect any significant inhibition with IgG4 from CP (Figure 17G-H). These results are in agreement with data obtained on granulocytes degranulation.

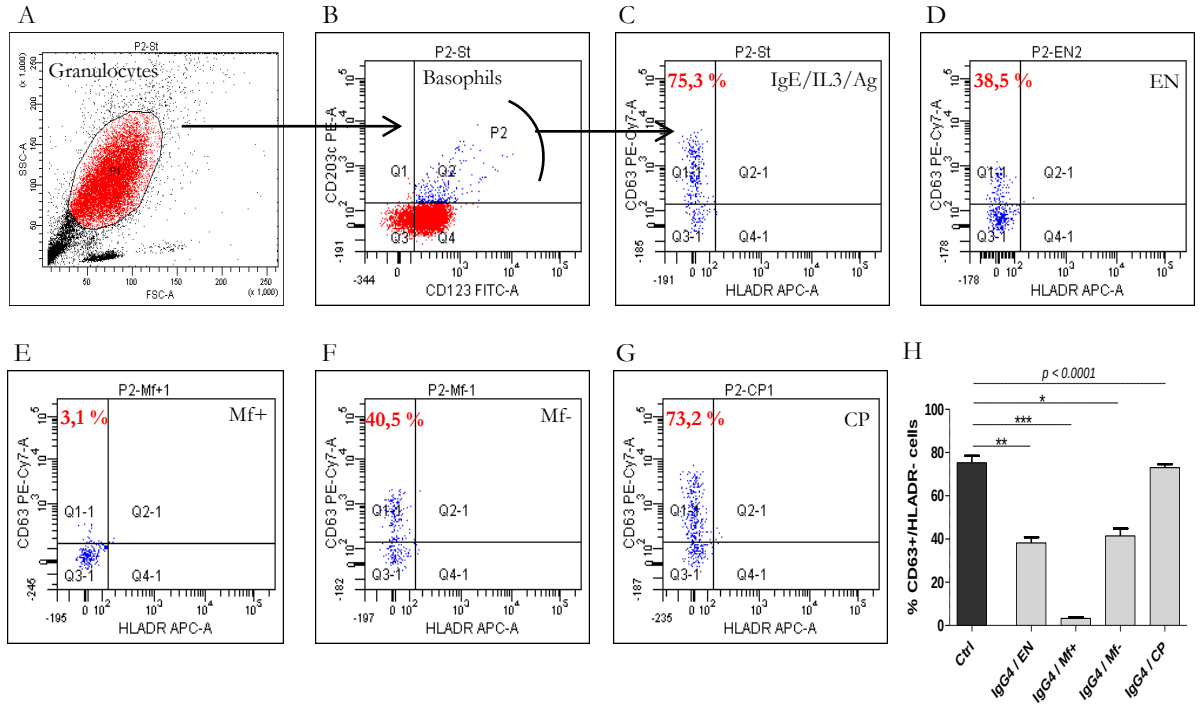

Figure 17: **IgG4 from EN, Mf+ and Mf- but not CP reduced basophil activation.** Granulocytes were isolated from healthy blood spenders (n=9) and 2 x 10^5 cells/well were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) alone (C) (dark bars) or in presence of 2.5 µg/ml of IgG4 antibodies (n=8) (grey bars) from EN (D), Mf+ (E), Mf- (F) and CP (G) for 18 hours. Basophils population was gated as CD203c+/CD123+ cells (B) from granulocytes population (A) and further analyzed for activation characterized by CD63+/HLADR- expression (C-H). Bars represent means ± SEM of the percentage of activated basophils. Asterisks show statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; **P < 0.01; ***P < 0.001.

In contrast, activation of eosinophils in presence of IgG4 antibodies produced a different result to that observed in neutrophils and basophils. Incubation of cells with IgG4 from EN,

Mf+, Mf- and CP did not alter eosinophils' activation (Figure 18D-H) compared to IgE and IL-3-stimulated control (Figure 18C, H). Although a slight inhibition was observed with IgG4 from Mf+, the effect remained not significant. This lack of effect of IgG4/Mf+ on eosinophils did not corroborate data on ECP release in granulocyte culture supernatants (section 4.1.5) as IgG4 from Mf+ reduced significantly the release of ECP.

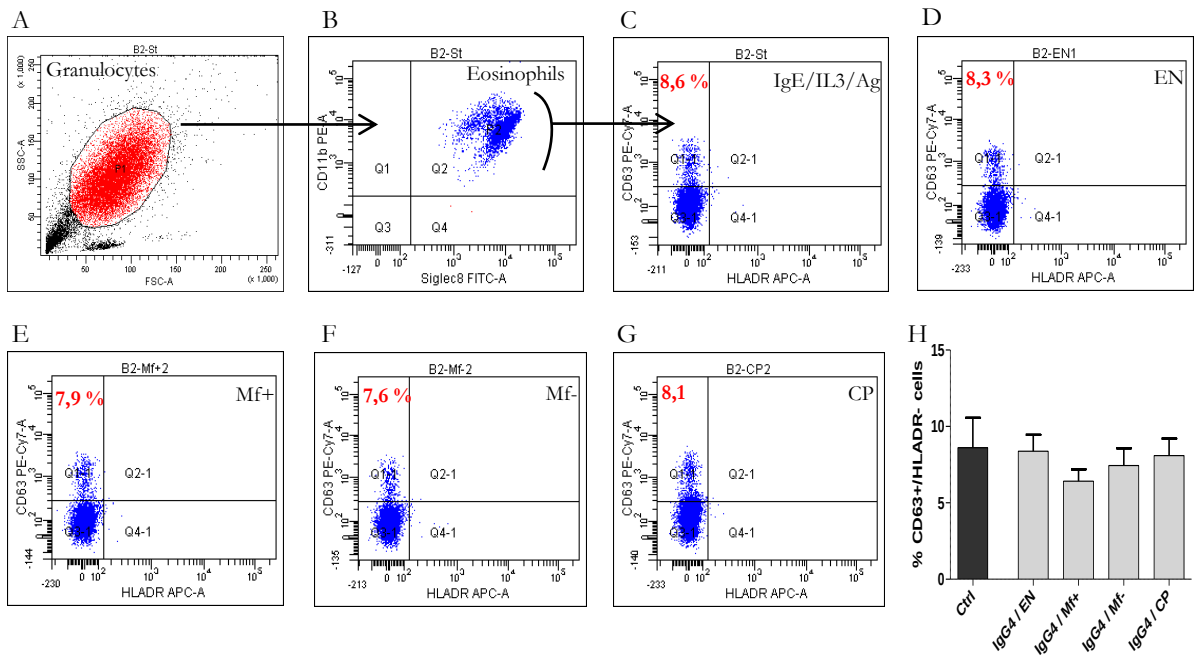

Figure 18: **IgG4 from EN, Mf+, Mf- and CP failed to inhibit eosinophil activation.** Granulocytes were isolated from healthy blood spenders (n=9) and 2 x 10^5 cells/well were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) alone (C) (dark bars) or in presence of 2.5 µg/ml of IgG4 antibodies (n=8) (grey bars) from EN (D), Mf+ (E), Mf- (F) and CP (G) for 18 hours. Eosinophils population was gated as CD11b+/Siglec8+ cells (B) from granulocytes population (A) and further analyzed for activation characterized by CD63+/HLADR- expression (C-H). Bars represent means ± SEM of the percentage of activated basophils.

Thus in LF infections, IgG4 antibodies associated with putative immunity and asymptomatic phenotype clearly modulate granulocyte functions by reducing neutrophils and basophils activation but failed to act on eosinophils.

4.2. Mechanisms of IgG4-mediated granulocyte inhibition in LF

4.2.1. IgG4 from Mf+ presented a higher affinity to granulocytes compared to IgG4/EN and IgG4/Mf-

To explore the mechanisms by which IgG4 interferes with granulocyte activities, we examined the ability of purified IgG4 antibodies from each group to bind on granulocytes (Figure 19). Therefore, granulocytes were cultured with IgG4 from all groups and immuno-

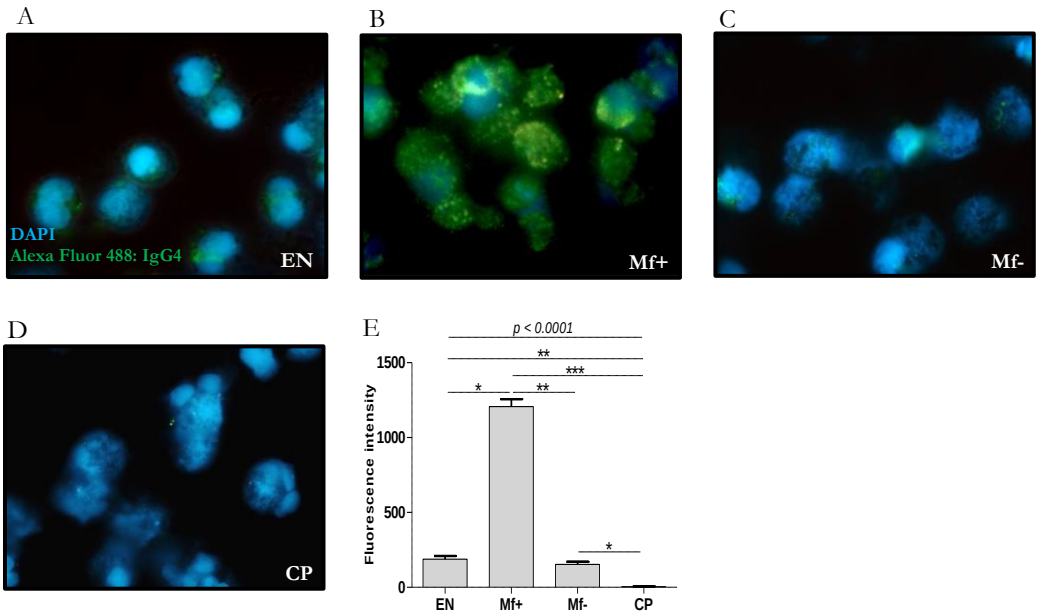

Figure 19: **IgG4 antibodies from Mf+ patients presented a higher affinity for granulocytes compared to IgG4 from EN and Mf-.** 2 x 10^5 cells/well of purified granulocytes from healthy blood spenders were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) and cultured in presence of 2.5 µg/ml of IgG4 antibodies from EN (A), Mf+ (B), Mf- (C) and CP (D) for 18 hours. The cells were then stained with DAPI (blue) and IgG4 binding on activated cells was revealed with Alexa fluor 488 labeled antibody (green). 6 fields were randomly chosen on the slides and the green fluorescence intensity, characterizing IgG4 binding on granulocytes, were measured. Original magnification x100. The median green fluorescence intensities are illustrated in E. A representative experiment of 3 is shown. Bars represent means ± SEM of the binding of IgG4 characterized as green fluorescence intensity. Asterisks indicate statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; **P < 0.01; ***P < 0.001.

fluorescence analysis was performed to reveal IgG4 presence on the surface of granulocytes. We could show that IgG4 from EN, Mf+, Mf- and CP differently bound granulocytes. While IgG4 antibodies from EN, Mf+ and Mf- were able to interact with effector cells (Figure 19A-C), no evidence of binding of IgG4 from CP to granulocytes was observed (Figure 19D). However, IgG4 from Mf+ presented a higher affinity for the cells in comparison to those from Mf- and EN as demonstrated by the increased mean fluorescence intensity (Figure 19E). These data are in line with the functional difference between IgG4 from CP and those from EN, Mf+ and Mf- observed in section 4.1.5.

As IgG4 from Mf+ preferentially bound to granulocytes, we next investigated which FcγRs are involved in this interaction.

4.2.2. IgG4 antibodies modulated granulocyte activities via a FcγRI and II-dependent mechanism

To determine the FcγRs involved in interaction with IgG4/Mf+, we used blocking antibodies against FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), which have been previously described to block the antibody Fc-mediated functions of different FcγR family members [265,266]. Blocking antibodies were added into granulocyte cultures prior to incubation with purified IgG4 antibodies. We observed that the blockade of FcγRI (Figure 20B, H) and FcγRII (Figure 20C, H) but not FcγRIII (Figure 20D, H) significantly reduced IgG4 binding to granulocytes compared with control (Figure 20A, H). In addition, relatively equal inhibition of IgG4 binding was measured when FcγRI or FcγRII was blocked. Interestingly, the capacity of IgG4 to bind granulocytes was completely abrogated when FcγRI and FcγRII were blocked simultaneously (Figure 20E, H).

As shown in figures 20F-H, the simultaneous blocking of FcγRI/III and FcγRII/III resulted in the exact observations as when using anti-FcγRI and anti-FcγRII antibodies alone, suggesting

clearly that FcγRIII might be playing a minor role in IgG4-mediated mechanism when FcγRI and FcγRII are actively involved in this mechanism. These results were also reflected when the activation of granulocytes in the presence of IgG4 and anti-FcγRs was measured. Indeed, the suppression of granulocytes by IgG4 antibodies was completely reversed when FcγRI and II were simultaneously blocked (Figure 20I). These findings suggest that IgG4-mediated granulocyte suppression in Mf+ patients involves FcγRI and FcγRII but not FcγRIII.

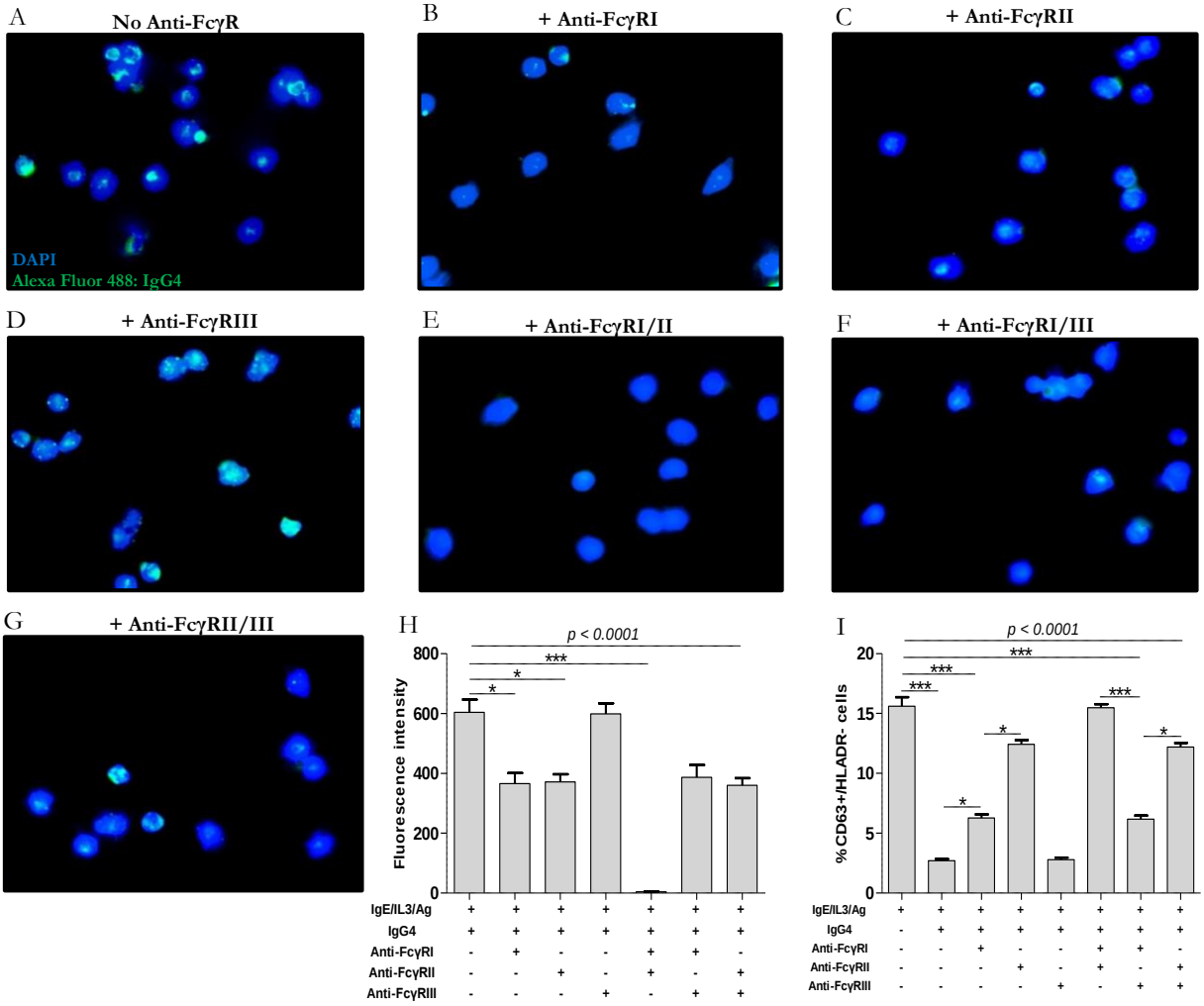

Figure 20: **Anti-inflammatory IgG4 antibodies modulated granulocyte functions via FcγRI and FcγRII.** Granulocytes from healthy blood spenders were purified and 2 x 10^5 cells/well were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) and incubated with either medium or anti-FcγRI, FcγRII or FcγRIII antibodies (A-G). Thereafter granulocytes were incubated with 2.5 µg/ml of affinity purified IgG4. The cells were then stained with DAPI (blue) and the presence of IgG4 was detected with anti-IgG Alexa fluor 488 antibody (green). 6 fields were randomly chosen on the slides and the green fluorescence intensity, characterizing IgG4 binding on granulocytes, were measured. A representative experiment out of 5 is shown.

Bars represent mean fluorescence intensities ± SEM (H) or the percentages of CD63+/HLADR- activated granulocytes (I). Asterisks indicate statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; ***P < 0.001.

To further explore the mechanisms of IgG4-mediated suppression, we next investigated the intracellular signaling pathways induced by IgG4 after binding on FcγRI and FcγRII.

4.2.3. IgG4 antibodies mediated differential phosphorylation of SHIP1, Src and Syk kinases

In order to elucidate the intracellular signaling pathways induced by IgG4 antibodies after interaction with FcγRI and FcγRII, we tested the following two hypotheses: 1) IgG4 may bind directly to an inhibitory receptor, such as FcγRIIb, and initiate the immunoreceptor tyrosine-based inhibitory motif (ITIM) pathways by inducing the phosphorylation of kinases such as SHIP1; 2) IgG4, after binding on FcγRI or FcγRIIa, may block the associated immunoreceptor tyrosine-based activation motif (ITAM) pathways characterized, in our settings, by the inhibition of the phosphorylation of the kinases Src and Syk and thereby granulocyte activation. To test these hypotheses, granulocytes were stimulated in the presence of IgG4 from all groups and cell lysates were submitted to SDS-PAGE. The phosphorylation of kinases of each pathway was assessed by western blot and phospho-flow cytometry using Src, Syk, SHIP1 and phospho-Src, Syk and SHIP1 antibodies. The results revealed that IgG4 mediated differential phosphorylation of Src, Syk and SHIP1 proteins (Figure 21). We observed an inhibition of the expression of the kinase Syk in presence of IgG4 from EN, Mf+ and Mf- but not CP. Interestingly, no phosphorylation of Syk was determined with IgG4 from all groups (Figure 21A). In contrast to Syk, the expression of Src was not inhibited but reduced in presence of IgG4 from EN, Mf+ and Mf- compared to the control. Moreover, these antibodies inhibited completely the phosphorylation of Src kinase. Interestingly, neither the expression nor the phosphorylation of Src was affected by IgG4 from CP (Figure 21A).

However, in the experimental system with western blot, SHIP1 protein was not detectable. We then investigated the effect of IgG4 on SHIP1 phosphorylation by phospho-flow cytometry, a more sensitive technique. We found that, the reduction of granulocyte activation correlated with increasing phosphorylation of SHIP1 (Figure 21B). In contrast to Src and Syk

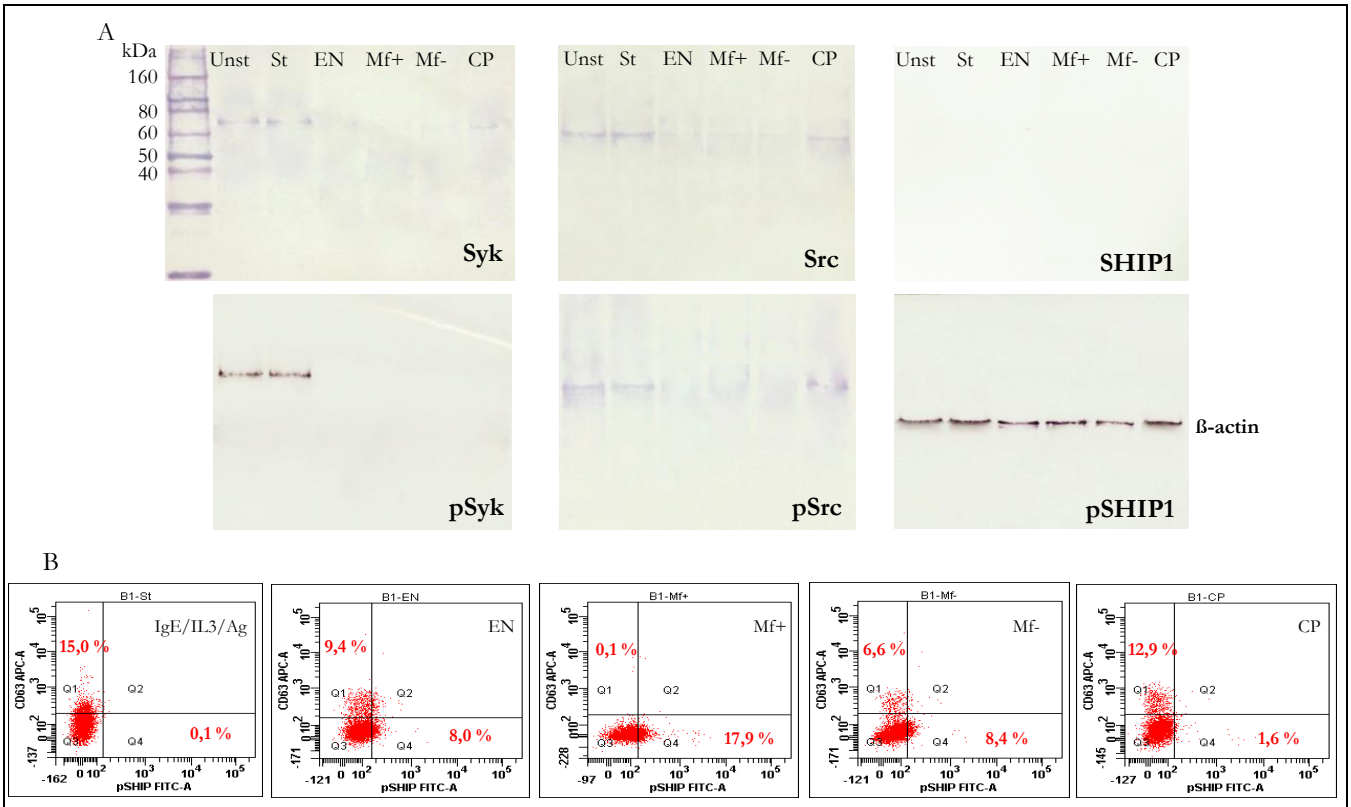

Figure 21. **IgG4 antibodies suppressed granulocytes by blocking the activation of Src and Syk and inducing inhibition via SHIP1 pathway**. Granulocytes from healthy blood spenders were purified and 1 x 10^6 cells/well were stimulated with IL-3 (2 ng/ml), IgE (25 ng/ml), and Brugia antigen extracts (10 µg/ml) and incubated with IgG4 antibodies from EN, Mf+, Mf- and CP for 2 hours. Then cells were lysed and total protein extracts were examined by western blot for phosphorylated and unphosphorylated products of the FcγR activation (Src and Syk) and inhibition (SHIP1) signaling pathways (A). The phosphorylation of SHIP1 was determined by phospho-flow cytometry as it was not detectable by western blot. Therefore, after incubation with IgG4, granulocytes were intracellularly stained for the phosphorylation of SHIP1 expression (B). Data are representative of 3 independent experiments.

kinases, the binding of IgG4 from EN, Mf+ and Mf- to granulocytes increased SHIP1 phosphorylation. We also detected a higher induction of SHIP1 phosphorylation in presence of IgG4 from Mf+ compared to EN and Mf-, which contrasts with lower phosphorylation of

SHIP1 with IgG4 from CP. (Figure 21B). These data indicate that IgG4 functions by blocking the activatory cascades through Src and Syk kinases and activating the inhibition signal via SHIP1 phosphorylation.

4.3. Modulation of complement activation in LF

4.3.1. Complement first component C1q expression did not vary in EN, Mf+, Mf- and CP but level of CIC-C1q increased in Mf+ plasma

C1q is the first component of the complement system. A classical function of C1q is to initiate complement activation and consequently its regulation impacts on complement functions. Previous studies reported the down-regulation of complement during helminth infections [79,220,222,232] but still the mechanisms sustaining this immune evasion are unclear. Helminth worm's derived products as well as host immune modified products have been evoked [79,247,267]. To study the involvement of IgG4 antibodies in complement inhibition during lymphatic filariasis, we first defined the C1q expression in plasma samples from EN, Mf+, Mf- and CP individuals by ELISA. As shown in figure 22A, no significant differences of the levels of C1q were observed between groups. Circulating Immune Complexes (CIC) composed of antigens, immunoglobulins and complement components are major contributing factors in the complement functions but also in the development of pathology in LF. CIC are potent activators of the complement system. We next investigated the levels of CIC, irrespective of a given immune complex, in plasma from different groups that interact with complement first component C1q (Figure 22B) by performing an enzyme immunoassay (EIA) as described in section 3.2.3.2. We observed that Mf+ patients exhibited significantly increased levels of CIC bound to C1q in comparison to EN and Mf- individuals. In contrast, relatively low levels of CIC-C1q were associated with CP patients reflecting, most likely, a relatively reduced antigen levels in the circulation.

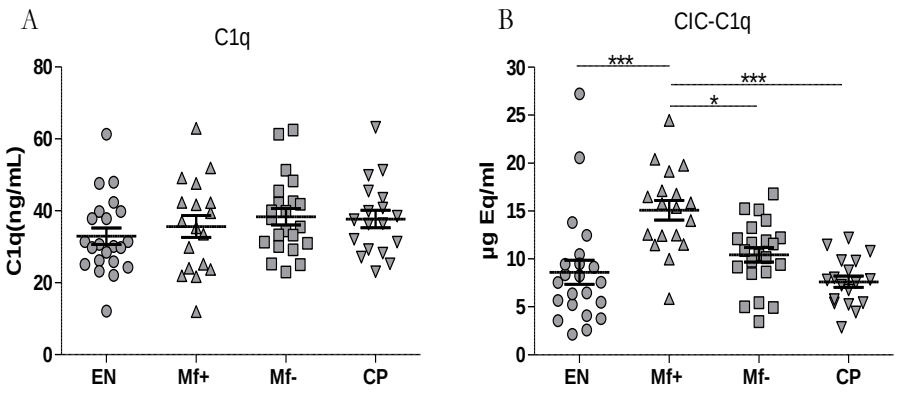

Figure 22. **Levels of C1q-bound circulating immune complexes (CIC) increased in Mf+ compared to EN, Mf- and CP**. C1q levels in plasma samples from EN (n=22), Mf+ (n=18), Mf- (n=22) and CP (n=18) were determined by ELISA (A). Then the levels of C1q-bound CIC were assayed by using CIC-C1q enzyme immunoassay (B). Bars represent means ± SEM of the concentration of C1q and CIC-C1q. Asterisks indicate statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; ***P < 0.001

4.3.2. Mf+ plasma displayed IgG1, IgA and IgE antibodies with reduced binding capacity to C1q whereas IgG4 exhibited very low capacity to bind C1q

CIC aggregates are mostly composed of IgG and IgM immunoglobulins. As Mf+ patients displayed higher levels of CIC bound to C1q, we are next interested in how the immunoglobulin isotypes in plasma of different groups interact with C1q. To address this question, we analyzed the capacity of each IgG subclass antibodies as well as IgA, IgE and IgM from EN, Mf+, Mf- and CP plasma samples to fix complement element C1q. Therefore, recombinant human C1q was coated at the concentration of 1µg/ml. The plates were thereafter incubated with plasma samples from different individuals and binding of plasma immunoglobulins was revealed with biotin-conjugated anti-IgG1-4, IgA, IgE and IgM antibodies. Data in figure 23 depict the binding capacity of each antibody to C1q expressed as arbitrary units (AU) relative to a standard serum arbitrarily set at 5 AU. The results indicated that IgG1 (Figure 23A), IgG2 (Figure 23B), IgA (Figure 23E) and IgE (Figure 23F) from patients differently fixed C1q. In contrast no differences could be observed in the binding

capacity of IgG3 (Figure 23C) and IgM (Figure 23G). Whereas the affinity of IgG1, IgG2 and IgG3 to C1q was higher, IgA, IgE and IgM displayed low affinity to C1q. The results also revealed the inability of IgG4 to fix complement (Figure 23D) as demonstrated elsewhere [29,111,208,268]. As represented in figures 23A, B and F, the binding capacity of IgG1, IgG2 and IgE from Mf+ individuals was significantly reduced than those of Mf- whereas IgG1, IgG2 and IgA showed high affinity to C1q in CP patients.

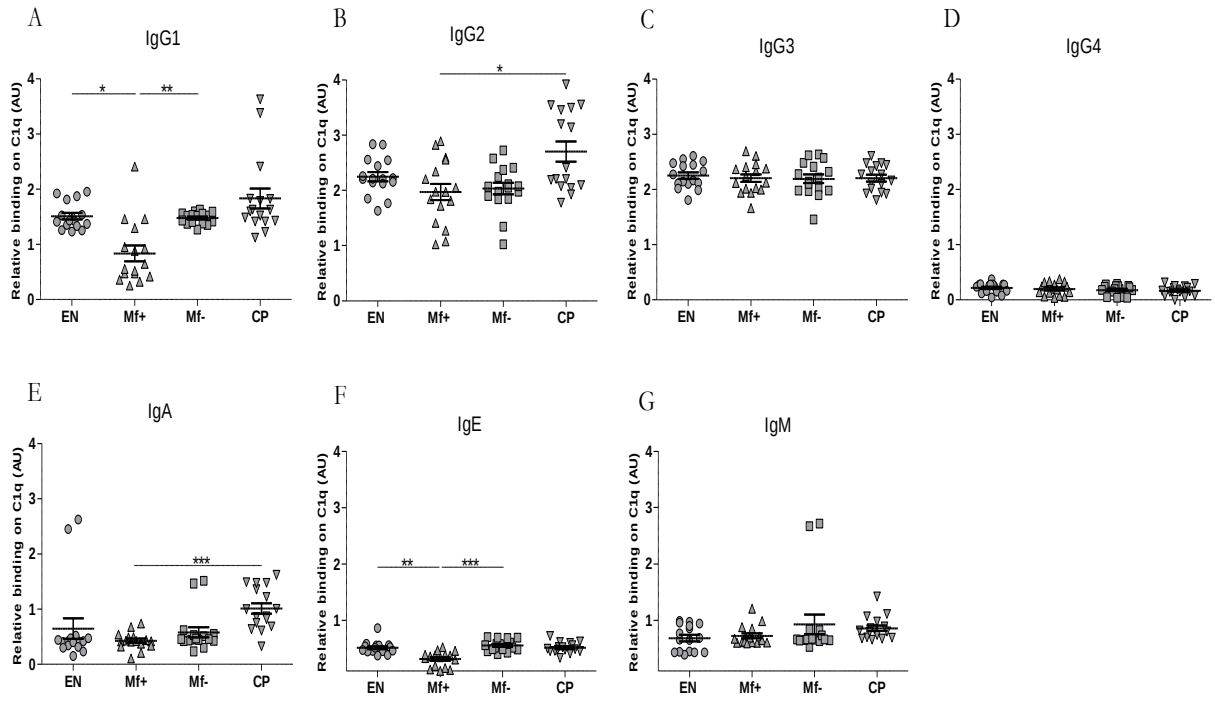

Figure 23. **IgG1, IgG2, IgA and IgE from LF patients differently fixed complement C1q**. IgG1 (A), IgG2 (B), IgG3 (C), IgG4 (D), IgA (E), IgE (F) and IgM (G) from EN (n=8), Mf+ (n=8), Mf- (n=8) and CP (n=8) plasma were tested for their ability to bind plate-coated complement C1q by ELISA. Results are expressed as arbitrary ELISA units (AU). Bars represent means ± SEM of the relative binding of immunoglobulins to C1q. Asterisks indicate statistical differences (Kruskal-Wallis test with a Dunn's nonparametric post-hoc) between the groups. *P < 0.05; **P < 0.01; ***P < 0.001

These results demonstrate that the ability of pro-inflammatory antibodies to fix C1q is reduced in Mf+ patients and therefore this might impact on complement activation in those patients.

4.3.3. Depletion of IgG4 from Mf+ plasma increased the binding capacity of IgG1 and IgG2

IgG4 is characterized with unique structural features, compared to other subclasses. IgG4 has been shown to bind other IgGs, in particular IgG1, via Fc-Fc interactions [269,270]. This may prevent other antibodies to exert their Fc-mediated immune functions. To study the implication of IgG4 antibodies in the reduced C1q-binding capacity of IgG1, IgG2 and IgE observed in above section, IgG4 was depleted from plasma from EN, Mf+, Mf- and CP and IgG4 negative plasma collected. Then IgG1, IgG2 as well as IgE from IgG4 negative plasma were tested for their ability to fix complement C1q (Figure 24). We could demonstrate that, after IgG4 removal from plasma, the C1q binding capacity of IgG1, IgG2 and IgE from EN and CP did not significantly change whereas those from Mf+ and Mf-, in comparison, were affected. A significant increase of C1q binding capacity of IgG1 and IgG2 was observed when IgG4 antibodies were removed from plasma of Mf+ patients (Figure 24A, B). Similarly we remarked an increase of C1q binding with IgG2 but not with IgG1 in Mf- patients. However IgE affinity to C1q did not change in Mf+ as well as in Mf- patients and remained lower (Figure 24C).

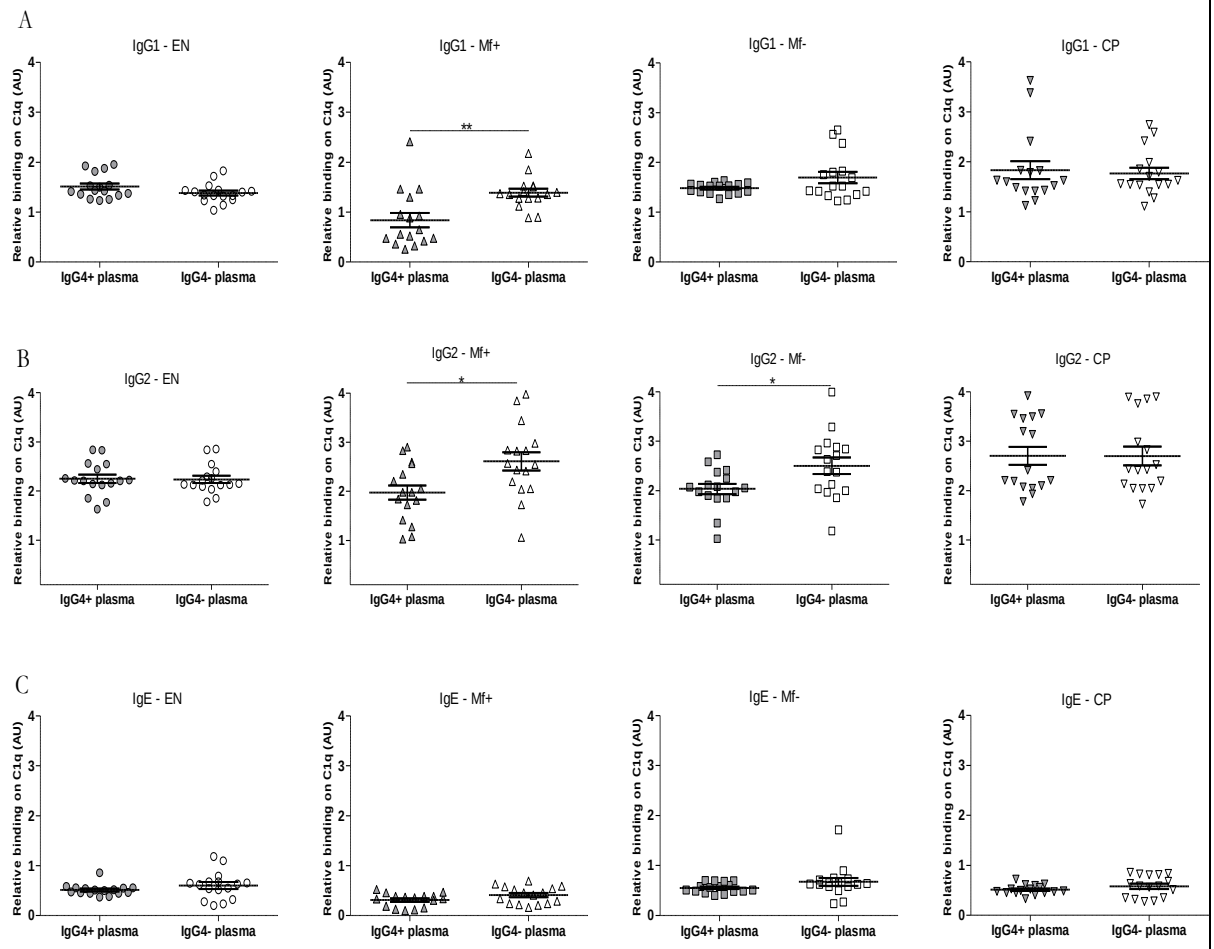

Figure 24. **Removal of IgG4 from plasma of Mf+ and Mf- enhanced the fixation capacity of IgG1 and IgG2 to complement C1q**. IgG1 (A), IgG2 (B) and IgE (C) from EN (n=8), Mf+ (n=8), Mf- (n=8) and CP (n=8) plasma were tested for their ability to bind plate-coated complement C1q by ELISA after removal of IgG4 antibodies. Results are expressed as arbitrary ELISA units (AU). Bars represent means ± SEM of the relative binding of immunoglobulins to C1q. Asterisks show statistical differences (Mann-Whitney test) between the two groups. *P < 0.05; **P < 0.01.

These data suggest that IgG4 might be this factor that hinders pro-inflammatory antibodies to fix complement and thereby might prevent indirectly complement activation by other antibodies.

5. DISCUSSION

5.1. Modulation of granulocyte activation and functions in LF

5.1.1. Patent filarial infection and putative immunity are associated with impaired granulocyte-related effector mechanisms

The pathology of lymphatic filariasis results from the complex interplay between the pathogenic potential of the parasite, the host's immune response and collateral bacterial and/or fungal infections. The isotype and level of antibody produced during the host's immune response play an important role in the outcome of helminth infections [52,172,271,272].

To initially characterize the antibody expression in the different clinical phenotypes associated with LF, the presence of antibody isotypes were measured in the plasma of EN, Mf+, Mf- and CP individuals. In comparison to EN, Mf- and CP groups (Figure 8D), we detected higher levels of IgG4 in Mf+ individuals. Mf+ patients are known to display low allergic and autoimmune diseases and our results confirm previous data suggesting that prominent IgG4 expression is characteristic of filarial asymptomatic infections [273] and the beneficial role attributed to IgG4 in allergic and autoimmune diseases. Furthermore, Mf- and CP patients are associated with higher plasma IgE levels. The elevated levels of IgG1 in EN and CP and increased expression of IgE and IgG2 found in patients with chronic pathology might be relevant for the clearance and destruction of microfilariae and adult worms. Filaria-specific IgE was shown to participate in the elimination of microfilariae and adult worms [274,275] but this isotype is also associated with pathology in LF [28,52,66,163]. Interestingly, EN and CP individuals presented increased IgG1 antibody levels but differ by IgE production, highly expressed in CP patients. These data suggest that IgG1 plays an important role in worm clearance. In line with this observation, Murthy and her group

detected high levels of IgG1 and IgG2 reactive to F6, a pro-inflammatory molecular fraction isolated from *B. malayi* adult worm, in serum of EN and CP individuals [276]. In addition, the group observed that the immunization with F6 intensively upregulated IgG1 and IgG2 in mouse and eliminated the infection [277]. Our data also indicated that high IgA levels are expressed in EN plasma compared to Mf+, Mf- and CP. This finding raises questions about the precise role of IgA in LF and suggests that IgA is likely to be involved in the protective immunity to LF.

Since Mf+ patients expressed high levels of IgG4 and IgG4 is known to display anti-inflammatory properties, and because granulocytes are critical for parasite elimination [112,113], we tested the ability of plasma from EN, Mf+, Mf- and CP to modulate granulocyte activation and degranulation upon stimulation with Brugia antigen, IL-3 and anti-IgE. Whereas plasma from NEC, Mf- and CP patients presented no effect on granulocytes in terms of activation and mediator release, those from EN and Mf+ significantly inhibited granulocyte functions (Figure 9C, D), suggesting that immune suppressive mediators are present in significant amounts in the plasma of individuals with patent infection and those with putative immunity. These observations are in line with findings of Mohapatra et al., indicating that plasma of asymptomatic individuals (Mf+) in contrast to those of CP mediated suppression of mitogen-induced proliferation of human PBMCs [278]. Bennuru et al. further demonstrated that sera from CP patients promoted the proliferation of lymphatic endothelial cells whereas those of EN suppressed this proliferation [24]. Plasma of Mf- and CP contain higher levels of pro-inflammatory IgG1-3 and IgE antibodies, known to be relevant for parasite clearance but are also associated with pathology development. The robust inhibition potential we observed when using plasma of EN contrasted with the potent inflammatory immune responses usually associated with putative immunity in LF endemic areas [25]. After depletion of IgG, we further demonstrated that non-IgG factors are responsible for

granulocyte inhibition by the plasma of EN (Figure 12A). These factors might include, as suggested by Bennuru et al., filarial-derived molecules or soluble angiogenic factors circulating in the plasma of EN [24]. Indeed angiogenic factors, such as angiostatin, expressed in human plasma [279,280], were shown in certain physiological conditions to inhibit the proliferation and migration of immune cells including neutrophils [281]. Another molecule that might be associated with granulocyte suppression by the plasma of EN is IgA. Our data reveal that elevated plasmatic IgA expression is restricted to EN (Figure 8F). Sahu et al. observed similar trends, when comparing the expression of filarial-specific IgA in LF endemic populations [282]. Recent investigations indicated that IgA is a multifaceted molecule that can display both pro and anti-inflammatory properties depending on the environment and can interact with FcαRI on the surface of eosinophils and neutrophils [283,284]. Our data also indicate that plasma from EN failed to inhibit the release of ECP but suppressed histamine and NE, and thereby related pathologies, suggesting that factors in the plasma of EN selectively suppress neutrophil and basophil but have no effect on eosinophils.

5.1.2. Lack of inhibitory properties of IgG4 antibodies on granulocytes correlated with pathological manifestations in LF

With the hypothesis that higher ratios of IgG4/IgG in the plasma of Mf+ might be responsible for granulocyte suppression, we next investigated the modulation of granulocyte activation and degranulation in the presence of purified IgG positive and negative fractions. The levels and specific reactivity of produced IgG antibodies in response to LF antigens have been extensively studied. However it remains unknown whether plasmatic IgG and especially IgG4 antibodies produced during human filarial infection can impact on the activities of effector cells such as granulocytes. In the present study, we purified IgG positive and negative fractions from plasma of EN, Mf+, Mf- and CP and tested the effect of these fractions on granulocyte activation and degranulation. We demonstrated that IgG positive fractions of Mf+

significantly suppressed granulocyte activation and the release of granule contents as shown by a significant reduction in the percentage of CD63 expressing cells and the amount of granule-associated immune mediators (histamine, ECP, and NE) (Figure 12B and F-G). Strikingly, IgG negative fractions from Mf+ presented a lower but significant suppressive capacity on isolated granulocytes. These findings suggest that factors other than IgGs might also be implicated in the ability of plasma from Mf+ individuals to suppress granulocyte activities. These factors might include, as suggested for EN, further filarial molecules, soluble angiogenic factors or IgA as discussed above. In addition, this suppressive activity by IgG negative fractions from Mf+ might also originated from a contaminating activity of the inhibiting factor present in IgG positive fractions. Further investigations are needed to characterize the molecules implicated in this IgG-independent granulocyte suppression.

With the hypothesis that IgG4 is responsible of the ability of IgG positive fractions of Mf+ to suppress granulocytes, we cultured *Bm*Ag/IgE/IL-3-stimulated granulocytes in the presence of affinity purified IgG4 antibodies from EN, Mf+, Mf- and CP individuals. We found a significant inhibition of granulocyte activation and degranulation in presence of IgG4 from EN, Mf+, and Mf-. These findings suggest that IgG4 antibodies from EN, Mf+ and Mf- have the same overall suppressive property on granulocytes. Thus, the alterations observed when using total IgG or crude plasma are probably due to differences in the ratios IgG4/total IgG as previously postulated [60,285]. However, when comparing the suppressive capacities of increasing concentrations of IgG4 from EN, Mf+ and Mf-, clear differences were observable. Indeed, at lower concentrations (1.25 µg/ml), purified IgG4 from Mf+ individuals presented a higher inhibition capacity when compared to those of Mf- and EN. These differences, however, disappeared when the concentrations were increased (5µg/ml) (Figure 14E). Since no difference was detectable in the purity of the IgG4 positive fractions and because IgG4 is known to present no allotypic variations, post-translational alterations including

glycosylation, fucosylation and sialylation (further discussed in section 5.2.1) must support the functional differences.

Interestingly we observed that only IgG4 antibodies from the group of patients with chronic pathology lacked inhibitory effect on granulocytes even when the concentration was increased. In addition IgG4 from this group failed to reduce the release of granule contents. As demonstrated in several studies, a lack of control on the release of secondary granule proteins such as ECP, EPO, EDN and NE may not only damage infectious worms but also tissues causing pathology and diseases [286,287]. This suggests that the pathological manifestations observed in CP patients might be a consequence, at least partially, of the lack of inhibitory properties of IgG4. Hence the importance of the regulation of IgG4 levels in LF.

5.1.3. EN, Mf+ and Mf- individuals displayed IgG4 antibodies with suppressive effects on neutrophil and basophil functions but not eosinophil's

To further investigate our understanding of the down-regulation of granulocytes by IgG4 antibodies produced during LF, we next analyzed which granulocyte subtypes are affected by IgG4 suppression effect. Several studies suggested distinct functions for the different granulocyte types (neutrophils, eosinophils and basophils). In LF, these cells are involved in inflammation, initiation of Th2 immune response against parasites and the promotion of the development and recruitment of immune cells [127,130,140,151,157]. A modulation of these functions may impact on the efficiency of the protection against helminth parasites. In our settings, we observed different effects of IgG4 on neutrophil, eosinophil and basophil populations. IgG4 from EN, Mf+ and Mf- actively impaired neutrophil and basophil activation and degranulation. This suppression of neutrophils and basophils may imply the impairment of critical immune functions against parasite mainly the initiation and maintenance of specific Th2 immune response, mediation of Th17 pathway of resistance to

parasite and protection against filarial secondary infection. These results indicate that IgG4 antibodies secreted during infections with filarial parasite might contribute to reduction of neutrophil and basophil mediated inflammation, which provides an immunological milieu for parasite survival. In contrast, IgG4 from EN, Mf+, Mf- and CP failed to reduce eosinophil activation. As shown by data on degranulation (Figure 15C), in Mf+ patients, IgG4 did not significantly reduce eosinophil activation but impeded their degranulation, which may support, when considering the inhibition of neutrophil elastase and histamine, the low rate of granule contents-mediated inflammatory diseases observed with LF asymptomatic individuals in endemic regions [10,54,62,288]. The diversity of IgG4-related effects on granulocytes might not only be supported by the functional differences observed between IgG4 molecules as suggested in section 5.1.2 but might also be linked to the distribution of FcγRs and FcεRs on the surface of neutrophils, eosinophils and basophils. FcRs are essential for immune cell functions. Neutrophils express FcγRI, FcγRIIb,c and FcγRIIIb whereas only FcγRIIa is consistently found on eosinophils [289,290]. FcγRIIa, FcγRIIb and FcγRIIIb are identified on basophils [207,291,292]. They express neither FcγRIIIa nor the high-affinity IgG receptor FcγRI [202]. The high affinity IgE receptor FcεRI is expressed consistently on basophils and at low levels on eosinophils and neutrophils [202,207,290]. FcγRI, FcγRIIa and FcγRIIIa are activating receptors when FcγRIIb is the only known inhibitory receptor. IgG4 binds to all of the Fcγ receptors with the exception of FcγRIIIb [203-206]. Thus the suppression of neutrophils and basophils by IgG4 may occur through direct interaction with the inhibitory FcγRIIb and/or as demonstrated by Kepley and colleagues, using a bi-specific antibody, by cross-linking of FcγRIIb and FcεRI which resulted in inhibition of IgE-mediated basophil activation [207]. These mechanisms seem to not be relevant when considering eosinophils since these cells are not known to express FcγRIIb, supporting the non-inhibition of eosinophil activation by IgG4 antibodies from EN, Mf+ and Mf- observed in our experimental

settings. These findings support evidence that IgG4 produced during LF infections inhibit granulocyte functions through the modulation of neutrophil and basophil-associated protection against filarial parasites.

5.2. Mechanisms of IgG4-mediated granulocyte inhibition in LF

5.2.1. IgG4 antibodies inhibit granulocyte activities via FcγRI and II

To investigate the mechanisms sustaining granulocyte inhibition by IgG4 from LF infected patients and EN suppressed granulocytes, we examined the ability of purified IgG4 from EN and all patients groups to bind to granulocytes. Our data indicated that IgG4 purified from EN, Mf+, and Mf- but not those from CP bind to granulocytes (Figure 19). However, IgG4 from Mf+ presented a higher affinity to granulocytes when compared with IgG4 antibodies purified from Mf- and EN. Difference in glycoforms of IgG4 could explain this variance in the binding capacity. EN and Mf- patients may express IgG4 glycoforms with moderate affinity to FcγRs on the surface of granulocytes while Mf+ individuals express glycoforms with higher affinity to FcγRs and consequently displayed the highest capacity to bind and suppress granulocytes. In contrast, chronic pathology patients may express IgG4 glycoforms with no affinity to FcγRs. Previous investigations have shown that a conserved glycosylation site at N297 in the CH2 domain of all IgGs is important for the structural conformation of the Fc region necessary for binding to FcRs and complement factors [293-295]. Differences in the glycosylation states may ultimately influence the effector pathways elicited by the Fc domain. In addition, fucosylation and sialylation for example are two extensively investigated glycan modifications of Fc domain that significantly modulate the affinity of Fc regions of antibodies to FcRs. In several health and disease settings, a shift toward certain Fab- and Fc-glycoforms of antibodies has been reported [296]. It is very likely that the degree of glycosylation differs

in the IgG4 molecules from EN, Mf+, Mf- and CP, which subsequently modulates their affinity to FcγRs.

We have also demonstrated that except CP group, IgG4 from all individuals suppress granulocytes after interaction with both FcγRI and FcγRII (Figure 20), confirming results of previous studies indicating that IgG4 binds to FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, and FcγRIIIA [203-206]. The activation FcεRI and FcγRs typically signal through an immunoreceptor tyrosine-based activation motif (ITAM), whereas the inhibitory FcγRIIb triggers signals via immunoreceptor tyrosine-based inhibitory motif (ITIM). Stimulation through ITAM pathway leads to pro-inflammatory signals leading to the destruction and clearance of antigens by phagocytosis, ADCC and promotion of antigen presentation. Bruhns et al. further demonstrated that IgG4 antibodies display a higher affinity for the inhibitory receptor FcγRIIb [289]. In our settings, this indicates that IgG4 antibodies may exert their suppressive properties via two distinct but complementary pathways. Suppressive IgG4 antibodies very likely bind to the inhibitory FcγRIIb and deliver an anti-inflammatory signal while impeaching pro-inflammatory antibodies to interact with FcγRI. These two mechanisms have been explored in cancer and allergen immunotherapies where IgG4 was shown to compete with inflammatory IgG1 for FcγRI binding on tumor specific monocytes [203] while IgG antibodies produced during subcutaneous allergen immunotherapy were demonstrated to mediate inhibition of basophil activation via a mechanism involving FcγRIIa and FcγRIIb [291].

5.2.2. IgG4 antibodies regulated the phosphorylation of SHIP1, Src and Syk kinases

To better define the mechanisms by which IgG4 suppressed granulocyte activation, we investigated the intracellular signaling pathways induced by IgG4 after binding on FcγRI and FcγRII. These receptors signal through important activation (ITAM) and inhibition motif

(ITIM), which, typically, represent the intracellular tail associated to the receptors. The cross-linking of activating FcRs, such as FcγRI and FcεRI, by immune complexes elicit phosphorylation of the ITAM by Src kinases, which further trigger the downstream signaling cascades, including mainly Syk kinase and phosphatidylinositol 3-kinase (PI3K) [179,182,297]. Consequences of the activating signaling include degranulation, ADCC and the initiation of inflammation. In contrast, when the inhibitory FcγRIIb is co-engaged with activating receptors, the tyrosine-phosphorylated ITIM recruits the SH2 domain-containing phosphatase SHIP1 which induces an inhibition signal or dampens the signal transduction elicited by activating FcγRs [181,297]. In this study we observed that the binding of IgG4 on FcγRI and FcγRII significantly increased the phosphorylation of SHIP1 but, in contrast, decreased and inhibited Src and Syk phosphorylation (Figure 21). This is supported by the observation that blocking FcγRI and FcγRII simultaneously, reduced completely granulocyte inhibition (Figure 20I). The binding of IgG4 on FcγRI did not induce granulocyte activation, reflecting the reduction of the phosphorylation of Src and Syk kinases we detected. This is in line with findings from Karagiannis et al., who documented in a cancer model that after binding on FcγRI, IgG4 did not increase Src phosphorylation in contrast to IgG1 [203]. They suggested that IgG4 did not induce a signal through the ITAM but, rather, blocked the binding of other IgGs on FcγRI and thereby the recruitment of Src, essential for the phosphorylation of the tyrosine in the ITAM. Indeed the recognized unique structure of IgG4 and its poor ability to activate effector cells, likely influences the binding pathway of the molecule to FcγRI and, thereby, the generation of the stimulus through the activation motif. In parallel the binding of IgG4 on FcγRII, most likely FcγRIIb, promoted a signal through the associated ITIM characterized by the phosphorylation of SHIP1. Furthermore post-translational modifications of IgGs from Mf+ individuals, such as glycosylation and sialylation, reported in helminth infections [58,111], are known to increase the affinity of IgGs to FcγRII. This

increases the phosphorylation of SHIP1, which leads to inhibition of granulocyte activation. Another mechanism may involve the effect of the co-engagement of FcγRII with activating FcγRI or FcεRI after simultaneous binding of IgG4 on both types of receptors. In our experimental settings, we used a cocktail of stimuli comprising IL-3 and IgE. It is well established that both IL-3 and FcεRI signal through Syk and phosphatidylinositol 3-kinase (PI3K) in granulocytes [180,298,299]. In relation to this finding, other studies demonstrated that once activated, the phosphatase SHIP1 removes the phosphate groups from the tyrosine residues of the ITAM [300] and, in addition, transforms the main product of PI3K, phosphatidylinositol-3,4,5-triphosphate (PIP3), into phosphatidylinositol-3,4-biphosphate (PIP2) [297,299] and thus dampens the signal transduction elicited by activating FcγRI and FcεRI. These results are consistent with reduced phosphorylation of Syk and dampened IL-3 and IgE-activation signal we observed. Altogether, IgG4 antibodies, and especially those from filariasis asymptomatically infected patients, may function by preventing the binding of pro-inflammatory antibodies (such as IgG1-3) on FcγRI/II and thereby blocking ITAM signaling pathways through Src and Syk. Simultaneously, IgG4 interacts with FcγRII-mediated SHIP1 phosphorylation which is known to dephosphorylate the tyrosine and PIP3 of the ITAM pathway induced by IL-3 and IgE and, in turn, inhibited the activation and degranulation of granulocytes.

5.3. Modulation of complement activation in LF

5.3.1. Patent infection in LF correlated with elevated levels of C1q-bound Circulating Immune Complexes (CIC)

The complement system is a major component of the innate immune system that plays a critical role during helminth infections [52,242,243]. The classical pathway of the complement system was shown to be principally activated by antibody-parasite antigen

complexes [52,229]. C1q, an integral part of the first component of complement (C1), triggers the activation process when it docks onto the Fc regions of antibodies within these immune complexes leading to parasite destruction [224,235]. The ability of helminth parasites to escape complement attacks by their derived products or induced molecules in host has been extensively explored [79,220,222,232]. These products and molecules were shown to bind directly to complement components or complement regulator proteins and block different stage of the complement activation system. However, whether IgG4 produced during helminth infections could hinder complement activation has not been to date investigated. As an initial step to characterize how IgG4 antibodies can interfere with the complement system during LF, we analyzed the expression of complement element C1q in plasma samples from LF infected and endemic normal individuals. No significant differences were observed between the four groups. The level of circulating immune complexes (CIC) is a marked feature of human lymphatic filariasis [28,301]. Several studies have reported significantly higher levels of CIC in LF patients with overt disease manifestations compared to Mf+ patients [302,303]. Another study demonstrated that microfilaria carriers harbored higher levels of CIC in comparison to endemic normal and patients with overt lymphatic pathologic changes [301]. We detected higher levels of CIC bound to C1q in plasma from Mf+ patients than EN, Mf- and CP individuals as measured by enzyme immunoassay (Figure 22B). Similar results were observed by Senbagavalli *et al*, where the elevated levels of CIC in clinically asymptomatic infected patients correlated with enhanced activation of granulocyte and complement classical and mannose-binding lectin pathways [301]. This observation may be associated to the presence of high levels of circulating Mf and bystander (non-filarial) antigens related to the hyporesponsiveness observed in Mf+ individuals. In contrast, the low levels of CIC-C1q observed in CP may result from the inflammatory state that characterized those patients and which leads to the elimination of antigens and parasites. In addition, as

suggested by Senbagavalli and colleagues, this observation might reflect the decrease of circulating immune complexes often seen in chronic pathology patients under anti-filarial treatment [304].

5.3.2. IgG4 antibodies are unable to fix complement but may prevent inflammatory antibodies to drive complement activation

We next investigated the comportment of each single antibody isotype from EN, Mf+, Mf- and CP by analyzing the affinity of each isotype with C1q (Figure 23). The results indicated that IgG1, IgG2, IgA and IgE from Mf+ patients presented a lower affinity to C1q compared to other groups whereas IgG4 antibodies from all groups, as reported elsewhere [29,111,208,268], are unable to interact with C1q. The binding of IgG1, IgG2 and IgG3 to C1q has been reported to play an important role in the activation of the classical pathway of the complement system [111,293,305]. These findings clearly suggest that, in comparison to EN, Mf- and CP groups, Mf+ patients presented an environment with reduced complement activation capacity. Based on this observation, the question was whether the differences in the capacity of the immunoglobulins to bind C1q directly correlated with the levels of IgG1, IgG2, IgA and IgE present in the plasma samples. As shown in figure 8, no difference was seen in the levels of IgG1 and IgA when comparing respectively Mf+ and Mf- and Mf- and CP plasma samples. Moreover, IgG2 and IgG4 levels were significantly higher in Mf+ compared to EN and Mf-. Thus, the quantity of these antibodies in the plasma probably does not affect their binding capacity to C1q.

In order to study the role of IgG4 antibodies in the differences observed in the binding capacity of immunoglobulins to C1q, we depleted IgG4 from the plasma by affinity chromatography and repeated the same experiment (Figure 24). We found that, after IgG4 was removed from plasma, the C1q binding capacity of IgG1 and IgG2 antibodies from Mf+

individuals significantly increased in contrast to previous results. The observation that IgG4 removal is associated with improvement of C1q binding suggests that IgG4 may be involved in the prevention of the interaction of IgG1 and IgG2 with C1q. As demonstrated by Rispens *et al* [269,270], IgG4, likely via Fc-Fc interactions, may bind to activatory IgG1 and IgG2 antibodies and inhibit their binding to complement C1q. This mechanism could eventually impact on other immune functions attributed to IgG1, IgG2. The inhibition of the complement by IgG4 may be beneficial for the host by limiting complement-associated pathologies.

5.4. Summary and conclusion

This thesis aimed to clarify the implication of IgG/IgG4 antibodies in the suppression of granulocyte and complement functions in the pathophysiology of lymphatic filariasis and the mechanisms underlying such suppression. The data of this study provide first evidences that prominent expression of IgG4 in asymptomatic Mf+ individuals is linked with inhibition of granulocyte functions. Indeed granulocyte activation and granule contents release were significantly inhibited by plasma of EN and Mf+ individuals. Our results reveal that this inhibition is associated with total IgG and non-IgG fractions of Mf+ patients but was abrogated when non-IgG factors were removed from EN plasma. Interestingly, the inhibitory effect of IgG in Mf+ is related to IgG4 antibodies. The results also indicate that, except in chronic pathology patients, IgG4 from EN, Mf+ and Mf- selectively reduced the activation of granulocyte neutrophils and basophils but not eosinophils after interaction with FcγRI and FcγRII. The binding of IgG4 on these receptors down-modulated the kinases Src and Syk but increased the phosphorylation of SHIP1; suggesting a functional difference between IgG4 isotypes from different individuals. This functional difference profoundly influences the activation of the complement during lymphatic filariasis infections. IgG4 is shown in this study to play an ambiguous role in complement suppression. It does not directly inhibit the complement but might hinder the activation of complement by IgG1 and IgG2 antibodies.

Collectively the data provide primary insights on the importance of both qualitative and quantitative modulation of IgG4 in the pathogenesis of lymphatic filariasis and its implication in the suppression of granulocyte and complement functions. Thus IgG4 antibody is an important target in lymphatic filariasis since the regulation of its level may be determinant for the development of the disease. In addition, learning from post-translational modifications of IgG4 induced by helminths may help to develop active therapies. However, the suppression of the different activation pathways of the complement system need to be more explored. Nevertheless, the inhibition of granulocyte in EN individuals raises some questions. Therefore, there is a need for further investigations into the nature and mode of action of non-IgG4 factors with suppressive effect in EN as well as the mechanisms underlying granulocyte suppression by IgG4 from EN and Mf- individuals.

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7. APPENDIX

Appendix A: Laboratory equipment

Equipment	Origin
BD FACS Canto™ flow cytometer	BD Biosciences, Heidelberg, Germany
Cellstar tubes (15 ml, 50 ml)	Greiner bio-one, Frickenhausen, Germany
Cellstar serological pipette, 5 mL, 10 mL	Greiner bio-one, Frickenhausen, Germany
Cellstar 96 well cell culture plate, sterile, U-bottom, with lid	Greiner bio-one, Frickenhausen, Germany
Centrifuge	Beckman Coulter, Krefeld, Germany
Costar Stripette, serological pipette,25 mL	Corning Incorporated, Corning, New York, USA
Cryo Tubes	Thermo Scientific, Roskilde, Denmark
Electrophoresis power supply EV243	Sigma Aldrich, Missouri, USA
Falcon serological pipette, 50 mL	Becton Dickinson Labware, Franklin Lakes, NJ. USA
Freezer (-20 °C)	AEG, Nürnberg, Germany
Freezer (-80 °C)	AEG, Nürnberg, Germany
Fridge	Liebherr, Biberach an der Riss, Germany

Glass Pasteur Pipette	Brand GmbH + CO, Wertheim, Germany
Incubator	Kendro, Langenselbold, Germany
Lamina Flow hood	Kendro, Langenselbold, Germany
MAGPIX, Xmap Technology	Luminex Corporation, Austin, Texas, USA
Micropipette Eppendorf Research	Eppendorf AG, Hamburg, Germany
Microplate, 96 well, F-bottom, PS, clear, High Binding	Greiner bio-one, Frickenhausen, Germany
Microscope (optical)	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany
Microscope (fluorescent)	Carl Zeiss, Thornwood, NY, USA
Mini orbital shaker	Stuart, Staffordshire, UK
Mini PROTEAN Tetra Handcast Systems	Bio-Rad, California, USA
Multichannel pipette (m300)	Biohit, Göttingen, Germany
Multipette stream	Eppendorf AG, Hamburg, Germany
Neubauer counting chamber	Marienfeld, Lauda Königshofen, Germany
Nitrocellulose Blotting Membrane	GE Healthcare Life Science, Solingen, Germany
Pipetboy acu	Integra Biosciences, Fernwald, Germany
Pierce Centrifuge Columns	Thermo Scientific, Rockford, USA

PS Microplates, 96 well, F-bottom	Greiner bio-one, Frickenhausen, Germany
Round-Bottom Tubes (5 mL)	Becton Dickinson Labware, Franklin Lakes, NJ. USA
Safe-Lock Tubes (0.5 – 2 ml)	Eppendorf AG, Hamburg, Germany
SpectraMax 190 Microplate Reader	Molecular Devices, California, USA
Tip One, pipette tips	Starlab Group, Hamburg, Germany
Trans-Blot Turbo, Transfer system	Bio-Rad, California, USA
Vacuum pump	ABM Greiffenberger, Marktredwitz, Germany
Vortex mixer (Minishaker)	VWR International, Darmstadt, Germany
Water bath	VWR International, Darmstadt, Germany

Appendix B: Chemicals and reagents

Reagent	Origin
Advanced RPMI 1640 (1X)	Life Technologies Corporation, Grand Island, NY, USA
Anti-Human CD11b PE	Ebioscience, San Diego, USA
Anti-Human CD15 PE	Ebioscience, San Diego, USA
Anti-Human CD16 FITC	Ebioscience, San Diego, USA

Anti-Human CD63 PE	Ebioscience, San Diego, USA
Anti-Human CD63 PE-Cy7	Ebioscience, San Diego, USA
Anti-Human CD66b FITC	Ebioscience, San Diego, USA
Anti-Human CD123 FITC	Ebioscience, San Diego, USA
Anti-Human CD203c PE	Ebioscience, San Diego, USA
Anti-Human HLADR APC	Ebioscience, San Diego, USA
Anti-Human HLADR FITC	Ebioscience, San Diego, USA
Anti-Human IgG4	Thermo Fisher Scientific, Rockford, USA
Anti-Human phospho-SHIP1 Ab (FC)	Stemcell Technologies, Köln, Germany
Anti-mouse IgG-AP	Bio-Rad, California, USA
Anti-mouse IgG (H+L)-Alexa Fluor 488	Thermo Fisher Scientific, Rockford, USA
Anti-rabbit IgG-AP	Cell Signaling Technology, Beverly, USA
Anti-rabbit secondary IgG-FITC	Abcam, Cambridge, UK
AP Conjugate Substrate Kit	Bio-Rad, California, USA
ß-actin Ab	Cell Signaling Technology, Beverly, USA
Bovine Serum Albumin, 2 $mg.mL^{-1}$	Thermo Scientific, Rockford, USA
Bovine Serum Albumin	PAA, Pasching, Austria
Calcium chloride	Roth, Karlsruhe, Germany

CaptureSelect Human IgG4 matrix	Thermo Scientific, Rockford, USA
Coomassie blue G	Cytoscelecton, Denver, USA
Dulbecco's PBS	Life Technologies Corporation, Paisley, UK
Fetal Bovine Serum, Standard Quality	PAN Biotech, Aidenbach, Germany
Fixation/Permeabilization Concentrate	Ebioscience, San Diego, USA
Fixation/Permeabilization Diluent	Ebioscience, San Diego, USA
Gentamycin (50 $mg.mL^{-1}$)	Life Technologies Corporation, Grand Island, NY, USA
Glycin	Sigma-Aldrich, Steinheim, Germany
HEPES	Sigma-Aldrich, Steinheim, Germany
Human anti-IgE mAb	Abnova, Taipei, Taiwan
Human Antibody Isotyping Panel 7-Plex Kit	EBioscience, Vienna, Austria
Human IgE mAb	Abcam, Cambridge, UK
Human IL3	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Human C1q	Sigma-Aldrich, Missouri, USA
L-Glutamine 200 mM (100X)	Life Technologies Corporation, Grand Island, NY, USA

Magic Mark XP, western standard	Invitrogen, California, USA
Normal Rat Serum	Ebioscience, San Diego, USA
Page Ruler, prestained Ladder	Thermo Scientific, Rockford, USA
Pancoll Human (1,077 $g.mL^{-1}$)	PAN Biotech, Aidenbach, Germany
Paraformaldehyde (PFA)	Merck KgaA, Darmstadt, Germany
Penicillin Streptomycin 10000 $Units.mL^{-1}$	Life Technologies Corporation, Grand Island, NY, USA
Permeabilization Buffer (10X)	Ebioscience, San Diego, USA
Phosflow Lyse/Fix Buffer	BD Bioscience, Heidelberg, Germany
Phosflow Perm/Wash Buffer	BD Bioscience, Heidelberg, Germany
Protein Assay Reagent	Cytoskeleton, Denver CO, USA
Protein G matrix	GE Healthcare, Freiburg, Germany
Purified anti-human CD16	Biolegend, San Diego, CA, USA
Purified anti-human CD32	Biolegend, San Diego, CA, USA
Purified anti-human CD64	Biolegend, San Diego, CA, USA
RBC Lysis Buffer (10X)	Ebioscience, San Diego, USA
Rotiphorese Gel 30	Roth, Karlsruhe, Germany
SHIP and P-SHIP1 rabbit Ab Syk and P-Syk rabbit Ab	Cell Signaling Technology, Beverly, USA

Src and P-Src rabbit Ab	
Sodium chloride	AppliChem, Darmstadt, Germany
Sodium dodecyl sulfate, ultra pure	Roth, Karlsruhe, Germany
Sodium hydrogen phosphate	Merck, Darmstadt, Germany
Tris buffered saline	Bio-Rad, California, USA
TEMED	Roth, Karlsruhe, Germany
TRIS	Merck, Darmstadt, Germany
Trypan blue 2 % in PBS	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Tween 20 EIA Grade	Bio-Rad, California, USA
VECTASHIELD-Antifade mounting medium	Vector Laboratories, CA, USA

Appendix C: Buffers and solutions

Culture Medium	500 mL RPMI 1640 10 % FCS (heat inactivated at 56 °C) 100 Units mL^{-1} Penicillin/Streptomycin 2 mM L-Glutamine 50 µg mL^{-1} Gentamycin
Coomassie Blue G	50 % methanol 30 % acetic acid

	0.05 % coomassie blue
Elisa blocking buffer	Assay diluent (5x), diluted 1:5 in distilled water
Elisa coating buffer	For 1 liter: weigh 8.4g of NaHCO3 Add 900ml distilled water Adjust the PH to 9.6 using KOH, Top to 1 liter
Elisa wash buffer	For 5 liters: weigh out 47.75g of PBS Fill up with distilled water Add 2.5ml Tween 80 Adjust pH 7.2-7.4
Elisa stop solution 2N H_2SO_4	For 500 ml: add 473ml distilled water to 26,6ml H_2SO_4
FACS Buffer	PBS / 2 % FCS
Fixation/Permeabilization	1:4 dilution of concentrate + diluent
PFA 4 %	4 g Paraformaldehyde in 100 mL PBS, pH 7.4
SDS-page: 4 % polyacrylamide stacking gel	Upper buffer 2.6 mL Acrylamide 1.33 mL ddH_2O 6.0 mL 10 % APS 100 µL TEMED 10 µL
SDS-page: 10 % polyacrylamide resolving gel	Lower buffer 4.16 mL Acrylamide 5.33 mL ddH_2O 6.3 mL 10 % APS 160 µL

	TEMED 16 µL
SDS-page: Lower buffer	1.5 M Tris 0.4 % SDS pH 8.8
SDS-page: Upper buffer	0.5 M Tris 0.4 % SDS pH 6.8
Towbin buffer	25 mM Tris base 192 mM Glycin 20 % methanol
Western Blot: wash solution	TTBS: TBS / 0.05% Tween 20
Western Blot: blocking solution	TBS / 3 % BSA
Western Blot: antibody buffer	TTBS / 1.5 % BSA

ERKLÄRUNG

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbst und ohne jede unerlaubte Hilfe angefertigt habe, daß diese oder eine ähnliche Arbeit noch keiner anderen Stelle zur Prüfung vorgelegt wurde und daß sie weder ganz noch im Auszug veröffentlich worden ist.

Bonn, den……

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Ulrich Fabien Prodjinotho

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