The influence of maternal Loa loa and Mansonella perstans infection on the distribution of Th1, Th17 and T regulatory T cell subsets in cord blood.

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Dedication

Gewidmet meinen Eltern

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Abbreviations used

APC	Antigen presenting cell
CBMC	Cord Blood Mononucleated Cells
CD	Cluster of differentiation
CERIL	Comité de Ethique Régional Indépendent de Lambaréné
CI	Confidence interval
CIRMF	Centre International de Recherches Médicales de Franceville
cm	Centimeter
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
e.g.	Exempli gratia
EDCTP	European and Developing Countries Trial Partnership
EDTA	Ethylenediaminetetraacetic acid
et al.	et alii
FACS	Fluorescence activated cell sorting
FCS	Fetal Bovine Serum
FMO	Flourescence Minus One
FSC-A	Forward Scatter Area
FSC-W	Forward Scatter Width
g	Gram
GCP	Good Clinical Practice
HBSS	Hanks' Balanced Salt Solution
HIV	Human immunodeficiency virus
HLA	Human lymphocyte antigen
ID	Identifier
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IPTp	Intermitted Preventive Treatment in pregnancy
km	Kilometer
L1-larvae	First stage larvae
L3-larvae	Third stage larvae
L. loa	*Loa loa*
LUMC	Leiden University Medical Center
m	Meter
M. perstans	*Mansonella perstans*
mf	Microfilariae
mg	Milligram

MHC	Major histocompatibility complex
min	Minute
MiPPAD	Malaria in Pregnancy Preventive Alternative Drugs
ml	Milliliter
mm	Millimeter
n.	Number
^{o}C	Degree Celsius
P.	Plasmodium
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
r	Pearson coefficient
RAPLOA	Rapis assessment procedure for loiasis
rpm	Rounds per minute
S. haematobium	Schistosoma haematobium
SD	Standard deviation
SP	Sulphadoxine-Pyrimethamine
SSC-A	Sidewards Scatter Area
SSC-W	Sidewards Scatter Width
TCR	T cell receptor
TGF-β	Transforming growth factor beta
T_h	T helper
TNFα	Tumor necrosis factor alpha
T_{reg}	Regulatory T cells
U	Unit
vs.	versus
WHO	World Health Organization
β	regression coefficient beta
μl	Microliter
μm	Micrometer

1 Introduction

This doctoral thesis is investigating the effect of in utero exposure to filaria antigens on the $CD4^+$ T cell distribution in cord blood. There is increasing evidence that immune priming of the fetus already starts in utero. Maternal infections can influence the developing immune system of the fetus independently of whether vertical transmission is taking place [1]. Therefore especially in the developing world, with high prevalence for chronic infections during pregnancy, the fetus is exposed to a variety of antigens through the infections experienced by its mother [1]. In certain territories a common infection also present in pregnant women is filariasis. The filarial parasite is rarely transmitted vertically, but it has strong immunomodulatory abilities and several studies have demonstrated evidence that it is leading to sensitization in utero [2, 3, 4]. Epidemiological studies have shown that children born to filaria infected mothers are more susceptible to filaria infection [2, 3, 4]. There is even evidence that this immune modulation can extend to third party antigens [1, 5]. For example it has been shown that children born to filaria infected mothers have a higher risk of mother to child transmission of human immunodeficiency virus (HIV) and a lower immune response to vaccination [4, 6, 7].

Currently it is not well understood how exactly the in utero exposure affects the cellular immune response. For this reason the present thesis questioned the influence of filarial infection during pregnancy on the $CD4^+$ T cell populations in cord blood.

1.1 Filaria infection in the developing world

Filariasis is an infection mainly occurring in the developing world and is also endemic in the Central-African country Gabon where this study was carried out. This leaves amongst others pregnant women at high risk for this infection [8].

While infections like malaria and HIV are of great interest in the scientific community filariasis is an infection less well studied and there is a lack in broad epidemiological data. It is estimated that world wide there are between 3-13 million people infected with *L. loa* [9] and 114 million may be infected with *M. perstans* [10]. *L. loa* is endemic to the rainforests of western and central Africa from a latitude of 8-10° North to 5° South. It is most common in the Republic of Congo, the Democratic Republic of Congo, Cameroon, Equatorial Guinea, the Central African Republic, Nigeria and Gabon. In a recent survey using the

rapid assessment procedure for loiasis (identified as RAPLOA), which combines a history of eye worms with the level of endemicity of the infection, it was estimated that nearly 30 million people live in high to intermediate risk areas with a prevalence of eye worm history exceeding 20% [11].

M. perstans infection is a disease not linked to a clear clinical picture and only very few studies have been carried out to evaluate the epidemiology of *M. perstans*. It has been estimated that 114 million people may be infected and as many as 581 million people in about 33 countries are at risk of infection in Africa alone. In endemic areas its prevalence during pregnancy can be up to 20% [1, 10]. *M. perstans* is endemic to a large portion of Sub-Saharan Africa, from Senegal to Uganda and south to Zimbabwe, as well as in Central and South America, from Panama to Argentina [10].

1.2 *Loa loa and Mansonella perstans*

Filariasis is a disease caused by tissue-dwelling nematodes (roundworms) of the superfamily Filaroidea. Currently 8 filarial nematodes that use humans as their definitive host are known. They can be differentiated into three groups according to the habitat of the adult worm in its vertebrae host. The group located in the lymph system contains *Wucheria bancrofti, Brugia malayi and Brugia timori*. The subcutaneous group includes *Loa loa, Mansonella streptocerca and Onchocerca volvulus*. The group living in the serous cavities holds *Mansonella perstans and Mansonella ozzardi*. *M. perstans* and *L. loa* are the two endemic species in Gabon and therefore the ones this thesis will focus on [12].

1.2.1 *Loa loa*

L. loa, often referred to as the African eye worm, was first described in 1770 by the french surgeon Mongin in the Carabean (Santo Domingo), who observed the worm passing a girls eye. The first microfilariae (mf) were only discovered in 1890 by Stephan McKenzie [13].

Life-cycle The infective larvae are transmitted to the host by the bite of an infected female fly of the *Chrysops species*. In the following 12 months these larvea develop into white threadlike adult worms, which migrate through the subcutaneous tissue (including the subconjunctiva from where the name eye worm originates) at a rate of up to 1cm/min. Adult worms grow to a size of 3-7cm

x 4mm and may live up to 17 years. In case of bisexual infections mf are produced and released into the bloodstream [14]. The mf produced by *L. loa* are sheathed and can be found in spinal fluid, urine, sputum, peripheral blood and in the lungs. Mf have diurnal periodicity and are found circulating in the peripheral blood during the day, but at night are in the vascular parts of the lungs and are non-circulatory [15]. This diurnal periodicity coincides with the feeding patterns of the principal vectors *Chrysops silacea and Chrysops dimidiata*. During another blood meal the mf can reenter the vector, loose their sheaths, penetrate the flies midgut and migrate to the thoracic muscle where they develop from the first stage larvae (L1-larvae) to the infective third stage larvae (L3-larvae). During the next blood meal these L3-larvae can again be transmitted to the next human host (Figure 1) [15].

Disease The majority of infected people in endemic areas are asymptomatic even though usually high levels of mf can be found in the blood. The symptoms are predominantly allergic reactions including pruritus, urticaria, and transient migratory swellings called Calabar swellings. All of those are mainly caused by the immunological reaction of the host. Symptomatic patients often without detectable mf in blood present with eosinophilia (often exceeding 3000/μl), hypergammaglobulinemia, increased serum IgE-levels and a strong humoral and cellular immune response to filarial antigens.

The most common complication is renal involvement including immune complex glomerulonephritis or mechanical trauma due to filtration of blood-born mf. Up to 30% of the patients present with hematuria or proteinuria, which may be transiently exacerbated by treatment. Before introduction of diethylcarbamazine in 1947 encephalitis was the most serious complication associated with the presence of mf in the cerebrospinal fluid. Other less common complications of loiasis include entrapment neuropathy, psychiatric disturbances, arthritis, lymphadenitis, hydrocele, pleural effusion, retinal artery occlusion, posterior uveitis, macular retinopathy, blindness, and endomyocardial fibrosis.

The first line treatment of *L. loa* recommended by the WHO is diethylcarbamazine. Alternative drugs are ivermectin and albendazol [16].

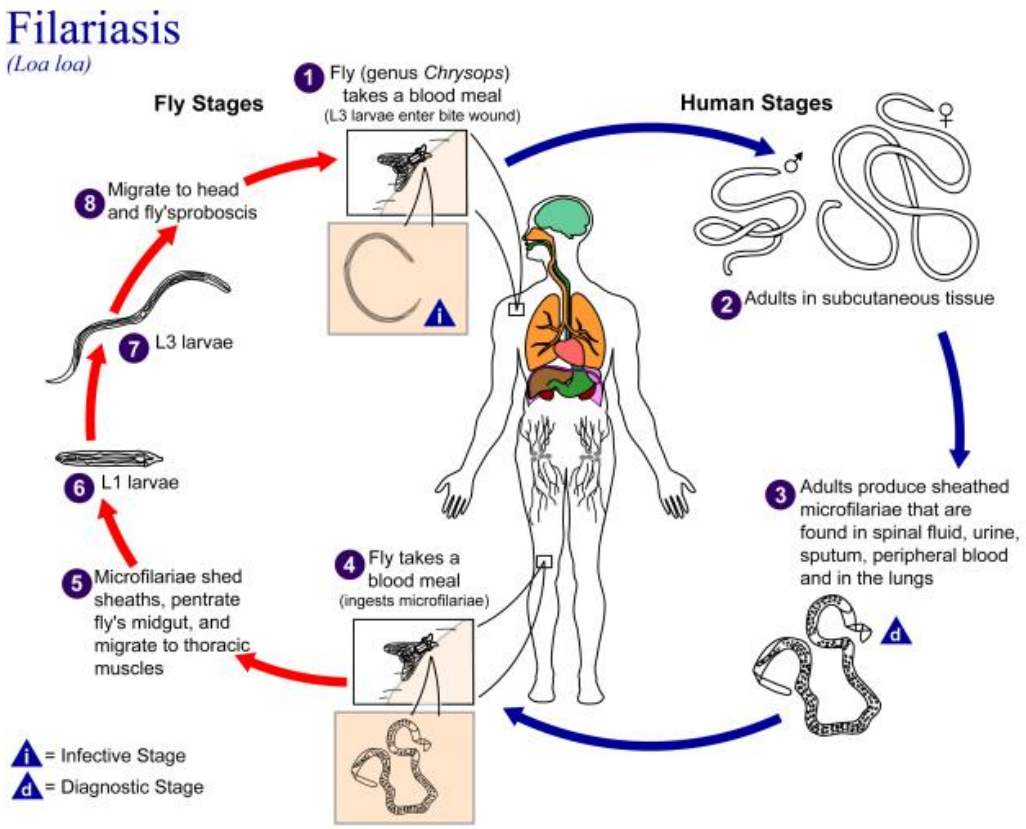

Figure 1: *L. loa* life-cycle [17]

1.2.2 *Mansonella perstans*

Life-cycle *M. perstans* is transmitted only by female biting midges of the genus *Culicoides*. When the midge bites a human the infective L3-larvae enter through the wound into the human. Over the following 9-12 months they mature to adult filariae. The adult male and female worms live in the serous cavities of the body (mainly peritoneal and pleural cavities less frequent in the pericardium) and can measure up to 3-8cm x 120μm. In case of bisexual infections the female worm can produce mf that are released into the bloodstream. Those mf are then ingested by the vector during the blood meal and migrate through it's stomach to the thoracic muscle where further development to initially non-infective L1-larvae and later on to infective L3-larvae takes place (Figure 2) [10, 14].

Disease Other as previously assumed *M. perstans* infections are not always asymptomatic and their presentation can be similar to that of *L. loa*. They can cause transient angioedema and pruritus of the arms, face and other bodyparts simular to the Calabar swellings of loiasis. Another common clinic presentation is urticaria. Less commonly observed are fever, headache, arthralgias and pain of the right upper quadrant. Rare complications are pericarditis, hepatitis, meningoencephalitis and neuropsychic disturbances. Occular granulomata and intraocular lesions as well as migration of adult worms through the subconjunctiva have been reported [14]. At the moment there is no ideal treatment for *M. perstans* infection. Different treatment strategies have been tested, but all with limited efficacy [10].

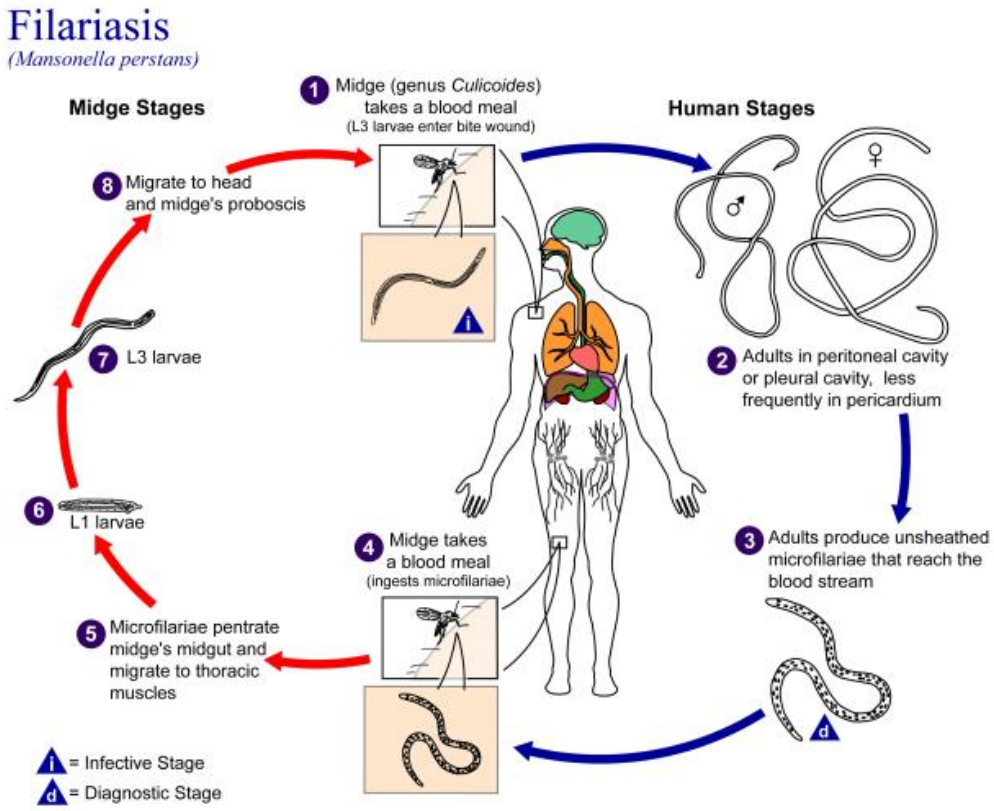

Figure 2: *Mansonella perstans* life-cycle [18]

1.3 Brief overview of the T cell mediated immunity

The immune defense to infection is often categorized into the innate immune response providing an unspecific but immediate protection against invading pathogens and the adaptive immune response providing a very specific and long lasting protection against pathogens, but takes a longer time to develop. The T cell response is part of the adaptive immune system [19].

The three major characteristics of the adaptive immune response are an exquisite specificity, the ability to generate a immunological memory and that it is highly adaptive and able to respond to unlimited numbers of molecules. For better understanding it can be separated into two major arms: the humoral immunity and the cellular immunity. The humoral immunity involves antibodies produced by B lymphocytes, while in the cellular immunity T lymphocytes play the central role [19].

1.3.1 The development of T lymphocytes

The T lymphocytes progenitor cells originate from the hematopoietic stem cells in the bone marrow from where they migrate to the thymus. In the thymus they undergo further maturation and the process of positive and negative selection, resulting in mature T lymphocytes. Every mature T cell expresses a unique T cell receptor (TCR) making it sensitive to one specific antigen. T cells with receptors incapable to react with the major histocompatibility complex (MHC) and auto-reactive T cells are detected and destroyed during the process of positive and negative selection. It is estimated that an individual processes 10^7-10^9 T cell clones each with an individual TCR and at least ensuring partial coverage for every antigen encountered. According to the protein chains that compose the TCR T cells can be divided into $\alpha : \beta$ (95%) and $\gamma : \delta$ (5%) T cells.

1.3.2 T cell mediated immunity

$\alpha : \beta$ T cells are of high importance for the immune response to viruses, fungi, parasites and intracellular bacteria. T cells regulate the activities of B cells, T cells and other cells participating in the immune response. They act as effector cells of the antigen-specific cell-mediated immunity and provide help for antibody production by B cells. During a process known as antigen processing and presentation the entering pathogen is processed into component peptides that bind to a structural framework on the cell surface known as human lymphocyte

antigen (HLA). While all nucleated cells have the ability to process and present antigens only a few are specialized antigen presenting cells (APC), these include dendritic cells (DC), macrophages and B-lymphocytes. The presented peptide/HLA-complex can then be recognized by individual T cells leading to their activation [19].

$\alpha : \beta$ **T lymphocytes and their subsets** T lymphocytes can be further categorized by their surface expression of either CD4 or CD8. The $CD8^+$ ("cytotoxic") T cells recognize antigenic peptides presented by HLA class 1 molecules (HLA-A, HLA-B and HLA-C) and are able to directly kill infected cells by producing pore-forming molecules called perforins or by triggering apoptosis. The $CD4^+$ lymphocytes help to suppress or to regulate immune responses by producing cytokines and providing co-stimulatory signals that support the activation of $CD8^+$ lymphocytes. They recognize the HLA class 2 molecules (HLA-DR, HLA-DP and HLA-DQ) [19].
$CD4^+$ cells can be subdivided into T helper (T_h) cells including T_h1, T_h2, T_h17 cells [20] and the regulatory T (T_{reg}) cells (Figure 3). There is evidence for the assumption that each T_h subset is involved in the immune response against a certain subset of microorganisms. T_h1 cells are of importance in the defense against intracellular microorganisms in general and mycobacteria in particular. T_h2 cells are of importance in parasitic infections and T_h17 cells seem to play an important role in the defense against extracellular bacteria, some fungi as well as parasitic infections [21]. The T_{reg} cells main function is to modulate or suppress the immune response [22, 23, 24]. On this context the exact circumstances leading to maturation of the subsets is not well understood. Whether a T_h1, T_h2, T_h17 or T_{reg} response develops is determined by antigens, the cytokine milieu and the APCs (Figure 3).

T_h1 cells The polarization of T_h0 cells to T_h1 cells is promoted by Interleukin-12 (IL-12) and the marker T_h1 cytokines are IL-2, Interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNFα). T_h1 cells promote the production of opsonizing antibodies (e.g. IgG1), the activation of macrophages and induce cellular cytoxity all leading to elimination of intracellular microorganisms. Additionally they are of importance in the delayed type hypersensitivity reactions. Overall the T_h1 cells are pro-inflammatory [25, 26, 27]. They can be classified by the presence of the transcription factor Tbet [28]. IL-12 leads to the activation of STAT4 that then activates Tbet. The activation of Tbet then induces the secretion

of IFNγ [29].

T_h2 cells The polarization of T_h0 cells to T_h2 cells is promoted by IL-4 and the major T_h2 effector cytokines are IL-4 and IL-13. Those are important for the IgE and IgG4 synthesis and stimulate eosinophil development to help mediate immunity against parasitic infections [30, 31]. Also they are the hallmark cells in atopic disease [25, 26, 27]. They can be classified by the presence of the transcription factor GATA-3 [28]. GATA3 induces the differentiation of T_h0 cells to T_h2 cells, while at the same time suppressing their differentiation towards T_h1 cells. It has also been shown to promote the secretion of IL-4, IL-5 and IL-13 by regulating gene expression [32].

T_h17 cells The polarization of T_h0 cells to T_h17 cells is promoted by IL-1 and IL-6 and the major T_h17 effector cytokine is IL-17. IL-17 induces the production of pro-inflammatory cytokines and chemokines and also recruits neutrophiles. The T_h17 cells are involved in the early response to extracellular pathogens and are also of importance in autoimmunity, tissue inflammation as well as the neutrophile predominant forms of asthma [33, 34, 35, 36]. They can be classified by the presence of the transcription factor RORγt [37]. That plays an important regulatory role in reducing apoptosis of undifferentiated T cells and promoting their differentiation to T_h17 cells [38].

Regulatory T cells The modulation of the immune response by the T_{reg} cells is achieved via different mechanisms including secretion of inhibitory cytokines (IL-10, transforming growth factor beta (TGFβ) and IL-35), cytolytic enzymes and suppressive molecules, metabolic disruption, expression of surface receptors that can influence dendritic cell function and an induction of target cell senescence [39, 40, 41, 42]. The dominant cytokines for T_{reg} induction are TGF-β and IL-2. These lead to an activation of STAT5 that leads to an activation of the transcription factor Foxp3 [43, 44]. The activation of Foxp3 leads to the secretion of the T_{reg} effector cytokines which are: IL-10, TGF-β and IL-35 In mice two different types of T_{reg} cells have been identified. The "natural" T_{reg} cells and the "induced" T_{reg} cells. In humans data on T_{reg} cells is less complete and the exact characteristics are still being determined. The natural T_{reg} cells are identified as "$CD4^+CD25^{high}$ and $Foxp3^+$" and appear to be the prominent subset in helminth infection [45]. This study focuses on natural T_{reg} cells as previous studies conducted by our group have unveiled the important role they play in filaria infection [46].

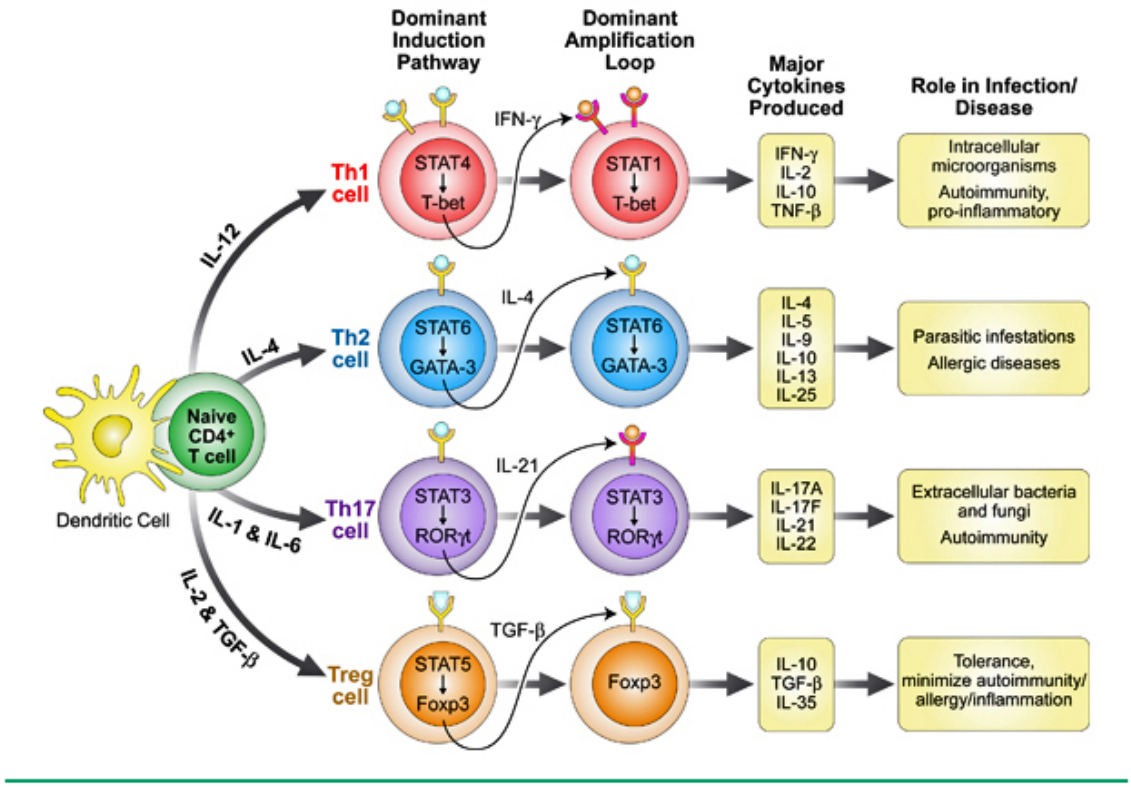

Figure 3: Th subsets [47]

1.4 T cells in newborns

Already at the end of the first trimester of gestation T and B lymphocytes and APCs can be detected in the fetal blood [1].

The T cell development in the fetus starts at about six weeks of gestation with the arising of the thymus from the 3^{rd} brachial arch. Over the next 2-3 weeks lymphoid cells migrate first from the yolk sac and the fetal liver later from the bone marrow to colonize the fetal thymus [48, 49]. Already at 12 weeks of gestation the thymus is organized into cortical and medullary regions and prothymocytes start dividing, rearranging their TCR genes and undergoing positive and negative selection to form T cells [50, 51, 52, 53]. These T cells migrate to the peripheral lymphoid organs at about 14 weeks of gestation [54].

In contrast to the humoral immunity the T cell mediated immunity is not transferred from mother to child. Therefore newborns rely exclusively on their own T cells. Still there are differences between the distribution and function of those T cells compared to adult blood:

In cord blood compared to adult blood the total lymphocytes (5400 cells/μl) as well as the total T cell numbers (3100 cells/μl) are increased. The numbers decline to adult level by 6-7 years of age. Also the $CD4^+$ T Cells contribute to a higher proportion of the T cells compared to the $CD8^+$ T cells. The $CD4^+$/$CD8^+$ ratio at birth is about 4.9:1 declining to a ratio of 2:1 (like in adults) by four years of age [55, 56].

During pregnancy the fetal cytokine responses are driven to a T_h2 phenotype. This is of importance as pro-inflammatory cytokines like from T_h1 cells can lead to abortion [57, 58]. Peripheral T cells in the fetus and the neonate are relatively immature. Fetal and neonatal APCs can prime antigen specific T lymphocytes, but their functional programs differ from those of adult cells restricting the development of effector T_h1 and possibly favoring the differentiation of regulatory T cells and T_h17 cells [1, 59].

1.5 Immunomodulation by Filariasis

Helminth infection has a strong effect on the immune system leading to a polarization towards T_h2 with high levels of the cytokines IL-4, IL-5 and IL-13 as well as high serum levels of IgE. Despite these initially strong T_h2 responses helminths are able to survive in their human hosts up to decades. This is believed to be possible through the induction of immunoregulatory mechanisms leading

to an hyporesponsiveness that prevents the elimination of the worm, but also reduces the symptoms caused by the inflammation. This hyporesponsiveness can also extend to third party antigens [5].

With adult filariae being able to live in their host up to 17 years, they are one of the helminth species with one of the highest average lifespan. To cause this chronic infection filariae had to develop different strategies to evade and down-regulate the hosts immune system. Comparing filaria-infected visitors to the endemic population it is conspicuous that visitors show more signs of inflammation, reduced parasite loads together with a pro-inflammatory T_h2 response [60]. This indicates that in the endemic population a down regulation takes place reducing the symptoms caused by an unaltered immune response.

Filariae induce regulatory T cells and alternatively activated macrophages, which are able to suppress T_h1 as well as T_h2 responses [2, 61]. This parasite specific anergy is primarily mediated by IL-10 and to a lesser extend by TGF-β and leads to lower type 1 (IL-2 and INF-γ) and type 2 (IL-5) cytokines [62]. Additionally the regulatory T cells can induce B cells to secrete IgG4. IgG4 molecules are capable of inhibiting IgE and IgG-mediated effector mechanisms leading to a lower pathology [63]. Another effect induced by filariae is the down regulation of DCs in the skin (Langerhans cells) by the L3-larvae. The L3-larvae impair the ability of Langerhans cells to express MHC class I and II, IL-8 and other molecules and leaves the cells inferior in their capacity to stimulate $CD4^+$ T cells [2, 64, 65].

Several studies have shown evidence that the immune modulation caused by filariasis already starts in utero as children born to filaria-infected mothers seem to have increased susceptibility to filarial infection. It has been shown that in utero exposure leads to a lower pro-inflammatory response, leading to fewer disease manifestations and allowing higher infection rates as well as higher parasite loads when the child gets infected [2, 3, 4]. In neonates exposed to filaria in utero specific IgE can be detected in cord blood. Additionally in-vitro studies have shown that their lymphocyte cytokine production to parasite antigen is similar to that of their mother [1]. Typical characteristics that were found analyzing cord blood comparing children from filaria infected vs. uninfected mothers are a lower proliferation of T cells in the umbilical blood, a higher level of the down regulatory cytokine IL-10 and higher levels of IgG4 [2]. A study carried out in Kenya showed a reduced T lymphocyte responsiveness to mf antigens in children born to mothers with lymphatic filariasis (no differences were seen in the stimulation with adult worm antigens) [63]. Another study carried out in Uganda indicated that maternal *M. perstans* infection during pregnancy was associated with stronger

IL-10 responses to mycobacterial antigens and to vaccination with tetanus toxin in exposed infants [6].

2 Materials and Methods

2.1 Study design, study area and study population

This cross-sectional study was carried out at the “Centre de Recherche Médical de la Ngounié” in Fougamou, Gabon from May 2011 till August 2011. “Centre de Recherche Médical de la Ngounié” was founded in 2006 and is a subunit of the "Centre de Recherches Médicales de Lambaréné", Gabon.

The Gabonese Republic is located in the western part of central Africa and is sharing borders with Equatorial Guinea in the northwest, Cameroon to the north and Republic of the Congo curving around the east and south. The west of Gabon borders the Atlantic with a coastal line of about 800 km (Figure 4).

Gabon covers a land area of 267 667 km^2 and an estimate from 2010 defines the population at 1.545 million with almost half the population (675 000) living in the capital Libreville. The population is composed of different ethnic groups of which the Fang represent the largest. Most inhabitants speak an ethnic language next to French, which is the official language.

Gabon has a tropical wet climate, with an average annual temperature of 26.6°C and a constant humidity of 80-90%. The precipitation of about 2600 mm is split between two rainy seasons, one short one from October until November, which is marked with a higher rain intensity and a longer one from February till May. 85% of Gabon is covered with rainforest of which only small parts were cleared for agriculture [66, 67].

Fougamou, a semi-rural town, is set in the province of Ngounié and lies along the west bank of the Ngounié river. It is placed on the National Road 1 between Lambaréné and Moulia (Figure 4). It has a population of approximately 4100 inhabitants [68]. The research unit is located next to the small hospital “Centre Médicale de Fougamou”. This hospital hosts four main departments: General Medicine, Stomatology, Pediatrics and a Maternity ward.

Fougamou as well as its surrounding areas are highly endemic for the human parasites *L. loa*, *M. perstans* and *P. falciparum*. A study published in 2011 by the Centre International de Recherches Médicales de Franceville (CIRMF) describes the overall prevalence in Gabon for *L. loa* at 22.4%, 10.2% for *M. perstans* and 3.2% for mixed infections [69]. CIRMF also published a paper describing the prevalence of malaria at 6.2% in adults [70].

The study took place in the framework of a study being performed in Fougamou and called "Malaria in Pregnancy Preventive Alternative Drugs" (MiPPAD). MiPPAD is a study funded by the European and Developing Countries Trial Partnership (EDCTP) and is executed on four study sites, being Benin, Gabon, Mozambique and Tanzania. The study is led by Prof. Clara Menéndez (Barcelona Centre for International Health Research, Spain) and aims to evaluate the safety, tolerability and efficacy of Mefloquin as an alternative drug to Sulphadoxine-Pyrimethamine (SP) for Intermittent Preventive Treatment in pregnancy (IPTp). By January 2012, the study ended recruitment, having enrolled a total of 4,734 pregnant women, after screening 17,947 women [71, 72].

In the context of the MiPPAD study 30 pregnant women with and without filaria infection were recruited and various blood samples were collected from the mothers and the cord of the newborn babies (details see below).

After finishing the sample collection in Fougamou in August 2011 the frozen samples were transported, for further analysis to the Leiden University Medical Center (LUMC), Leiden, the Netherlands. The parasitological examinations were carried out at the study site.

Figure 4: Map of the Republic of Gabon [73]

2.2 Study procedures

2.2.1 Recruitment of participants

This cross-sectional study was designed to examine the effect of maternal filaria infection on the frequency of $CD4^+$ T cell subsets in cord blood. For this purpose, two study groups were formed namely, filaria-uninfected and filaria-infected mothers. All participants were recruited within the MiPPAD study taking place in Fougamou.

Inclusion and Exclusion criteria To exclude factors that might have an influence on the T cells in cord blood, the following inclusion and exclusion criteria were established as shown in Table 2.

Table 2: Inclusion and Exclusion criteria

Inclusion criteria	Women included in the MiPPAD-Study Women who signed the informed consent for MiPPAD and MiPPADs axillary studies Women who agreed to an additional filarial screening Women in child bearing age Women from Fougamou and the surrounding villages
Exclusion criteria	Women or newborns with serious clinical conditions Cases of still-birth Women with acute malaria infection at time of delivery Placental malaria and malaria infection of the child Women giving birth to twins Birthweight lower than 2200 grams Women infected with HIV

2.2.2 Parasitological Examinations

All participants were screened for filaria, malaria, schistosomiasis and HIV. The filaria status was evaluated for all women qualifying for inclusion. For this a 2.8 ml Ethylenediaminetetraacetic acid (EDTA) tube was used to drain blood from the mothers whose date of delivery was expected within the next 2 months. Using the leuko-concentration method these blood samples were screened for *L. loa* and *M. perstans* [74].

The malaria, schistosomiasis and HIV screening was performed as a part of the MiPPAD-study. For Malaria thick smears were taken at inclusion, delivery and every time a woman presented with fever at an unscheduled visit. In this manner it was possible to record all the malaria episodes during the pregnancy from the moment the mother was enrolled in the MiPPAD-study. Besides the thick smear of the mother at delivery smears were also taken from the cord blood and the placenta to rule out placental malaria or malaria parasitemia in the newborn.

To evaluate the schistosomiasis status 10 milliliter (ml) of urine were collected from each woman and searched for eggs using the Urine Filtration Method recommended by the WHO [75].

Detection of microfilariae Pregnant women from the MiPPAD study, who were close to their calculated date of delivery (expected date of delivery within 2 months), were screened for *L. loa* and *M. perstans* using the Leuko- (Knott's-) concentration method. A modified Knott's concentration technique was applied as followed: 2.5 ml anticoagulant EDTA blood was collected from the mother and diluted 1:1 with 2% Saponin-solution in a conical tube in order to lyse the erythrocytes. The samples were placed on a rolling/tilting mixer for 15 minutes, centrifuged (5 min at 500 acceleration (g)) and then the supernatant was discarded. The pellet was placed on a microscope slide and covered with a cover slip. Then the whole slide was examined under the microscope (10x objective). The density of mf was calculated dividing the number of mf counted by the amount of original blood used resulting in the number of mf per ml blood. The parasite species were identified by their size and by the absence (*M. perstans*) or presence of a sheath (*L. loa*). If needed toluidine-blue was used to make the mf more visible [74].

Detection of *Plasmodium falciparum* To determine the malaria parasitemia a thick smear was preformed using the Lambaréné method [76]. The study patients

were pricked into their fingertip with a one-time use lancet. The first drop was discarded and then 10 μl of blood were taken from the fingertip with a pipette and distributed on a 2x1 cm area on a microscope slide. After drying the slide was coloured with 10% Giemsa in buffered water at pH 7.1 for ten minutes. Afterwards, the slide was rinsed with water and air dried. The parasite counting was done using a light-optical microscope counting all parasites. The parasitemia was defined as the number of parasites per μl (total parasites counted divided by 10 equaled the parasites per μl). The same colored slide was checked for mf. When mf were found, the parasitemia was also recorded as parasites per μl.

Diagnostic of *Schistosoma haematobium* At study inclusion 10 ml of urine were collected in a single use cup to screen the mothers for Schistosoma haematobium infection. A polycarbonate screen membrane filter was put in a Plastic Swinney filter holder and 10 ml of urine were pressed through the filter-system using a 10 ml syringe. Next the filter was carefully removed from the filter-system, laid on a microscope slide and the whole filter was examined for schistosomal eggs using the light microscope (10x objective). The sensitivity for this method is described at 50% [77].

Detection of HIV A quantitative Polymerase Chain Reaction (PCR) for HIV was performed at the study site to determine the viral load from the blood sample collected at enrollment to the MiPPAD-Study.

Treatment All participants were treated according to the MiPPAD-protocol and the guidelines at the study site. The women, who were found to be infected with *M. perstans* or *L. loa* during the screening, were offered a treatment with 400 mg Albendazole per day for seven days starting one month after delivery. All the treatments administered were recorded in the MiPPAD case report form.

2.2.3 Blood collection and hematological measurements

Cord blood collection 9 ml of heparinized cord blood was collected at delivery to perform immunological examinations. The blood was drawn from the umbilical vein attached to the placenta after the child was born and the umbilical cord has been cut and clamped. For this the umbilical cord was disinfected using 70% ethanol to rule out contamination with maternal blood.

Hematological measurements An EDTA tube of 1.2 ml blood was collected from the mothers at delivery and was used for a full blood count. This included erythrocytes, leukocytes, hematocrit, hemoglobin, platelets, lymphocytes, eosinophils, basophils, monocytes and neutrophils. The hemograms were generated using the ABX Pentra 60. During the MiPPAD-screening, 1.5 ml of capillary blood was collected for a HIV-test.

2.2.4 Immunological analysis

Cord Blood Mononucleated Cells (CBMC) Isolation The CBMC isolation was performed following the protocol "LUMC 002.2: Peripheral Blood Mononuclear Cell (PBMC) isolation" written by the department of parasitology at LUMC. The protocol was last reviewed by Yvonne Kruize in March 2011.

The blood processing took place within 24 hours after delivery and 4.5 ml anti coagulated cord blood from a heparinized vacutainer was used. The blood was centrifuged in the vacutainer and plasma was taken from the blood-cell pellet and frozen in a cryo vial. The blood cell pellet was then transferred to a 50 ml Falkon tube and diluted 1:1 with HBSS. After mixing 13 ml of 1.077-Ficoll were carefully placed under the blood/HBSS-suspension. The suspension was centrifuged without break at 1520 rounds per minute (rpm) for 25 minutes. The interphase between Ficoll and HBSS containing the CBMC was transferred carefully to another 50 ml Falcon tube. After two washing steps with HBSS the pellet was re-suspended in 5 ml of 10% FCS/ IMDM and an estimate of the number of cells isolated was calculated using the counting chamber. For this the cells were stained with Türk stain, filled into the counting chamber and the leukocytes in 25 squares were counted, including those touching the upper and the left border and leaving out those touching the lower and the right border. From this count, the number of cells per ml was calculated. The cells were then diluted to reach a concentration of 1 million cells per 200 μl.

Cell-fixation using eBio-Science The eBioscience fixation was performed following the protocol "LUMC 011.02: Ex-vivo fix-freezing cells by eBioscience" written by the Department of Parasitology at LUMC. This protocol was last reviewed by Linda Wammes in May 2010. 1 million of the isolated CBMC were added to a well on a 96-well-plate. The plate was centrifuged (1200rpm, 4 min) and the supernatant discarded. subsequently the cells were washed with PBS and after centrifugation (1200 rpm, 4 min) the supernatant was again discarded.

200 μl of eBioscience fixation buffer (1 part fixation/permeabilisation concentrate plus 3 parts fixation/permeabilisation diluent) was added to the pellet and the plate was incubated in the dark at 4°C for 60 minutes. After incubation 200 μl PBS was added, the plate was centrifuged (1200 rpm, 4 min) and the supernatant was discarded. 200 μl of freezing medium (RPMI: 10% FCS and 20% DMSO) was added, and the cells were transferred to a cryotube to be frozen at $-80\,^{\circ}\mathrm{C}$ freezer.

Thawing of cryopreserved Cells The thawing of cells was performed following the protocol "LUMC 020: Thawing of cryo-preserved fixed cells", written by the Department of Parasitology at LUMC. This protocol was last reviewed by Linda Wammes in March 2011. The samples were thawed, stained and measured by Fluorescence-activated cell sorting (FACS) at the same day. 5 ml tubes labeled with the donors number and containing 1 ml of thawing medium (RPMI with 10% FCS) at room temperature were prepared. The samples were taken out of the freezer and 0.5 ml of warm thawing medium was slowly added to each sample. The thawed cells were transferred to the corresponding 5 ml tube and centrifuged (1600 rpm, 5 min). The supernatant was discarded and the cells were re-suspended in 2 ml PBS. After centrifugation, the FACS staining was applied.

FACS-Staining For the FACS-staining a T cell panel was established for which surface and transcription markers were chosen to allow description of the cell population. The markers were chosen according to current literature and previous experience.

The FACS-staining was preformed using the protocols "LUMC 018.02: Intracellular FACS-staining for eBioscience-fixed cells" written by the Department of Parasitology at LUMC. This protocol was last revised by Kit Yeng Liu in June 2010.

For the staining of the samples a mix of fluorescent labeled antibodies Helios, RORγt, T-bet, CD25, GATA3, FOXP3 and CD4 was prepared in staining buffer (permealization buffer with 1% human FcγR-binding inhibitor) based on optimal working dilutions. The antibody dilutions used for a single stain were prepared in FACS buffer (PBS with 0.5% FCS and 2mM EDTA). Fluorescence Minus One (FMO) were prepared for the RORγt, T-bet, GATA3 and FOXP3 antibodies (Figure 5). All steps involving staining were carried out in the dark.

As some of the T cell antigens are intracellular antigens a permeabilization step was performed before staining. A V-bottom 96 well plate was prepared following a layout created in advance (Figure 6). Following the layout, 200 000 cells per donor

were added to corresponding wells. A cell-mix of all the filaria infected donors and a mix of the filaria uninfected donors only was prepared and added to the FMOs. Instead of cells compensation beads were added to the single stain wells in respect to the antibodies used (Mouse- or Rat-Ig). The plate was centrifuged (4 min, 1200 rpm) and the supernatant was discarded.

In order to stain the intracellular antigens 150 μl of permealization buffer (0.5 % Saponin in PBS) was added to the cells before the staining. The plate was centrifuged again and the supernatant discarded.

30 μl of staining mix, single stain or FMO was added to the corresponding wells and mixed by pipetting. The plates were incubated for 30 minutes in the dark at 4°C. After incubation 150 μl of permealisation buffer was added to the cells and the beads. The plates were centrifuged (4 min, 1200 rpm, 4°C) and the supernatant discarded. 50μl of FACS buffer were added to each well and the content was then transferred to 1.5 ml FACS insert tubes.

Channel	Flurochrome	Target	Isotype	Clone	End dilution
FL2	PE	RORγT	Rat IgG2a	AFKJS-9	1:150
FL3	PerP5.5	Tbet	Mouse IgG1	4B10	1:320
FL4	PE-Cy7	CD25	Mouse IgG1 k	2A3	1:160
FL5	*APC*				
FL6	Eflour660	GATA3	Rat IgG2b k	TWAJ	1:80
FL7	Eflour450	FOXP3	Rat IgG2a κ	PCH101	1:100
FL8	V500	CD4	Mouse IgG1 κ	RPA-T4	1:40
	CD16/CD32				1:100

Figure 5: Markers used for FACS-Analysis

Plate1: Panel 1 => T-Cells

	1	2	3	4	5	6	7	8	9	10	11	12
A	1=> 7366	2=> 7374	3=> 7375	4=> 7376	5=> 7377	6=> 7370	7=> 7373	8=> 7380	9=> 7381	10=> 7382		
B	11=> 7378	12=> 7384	13=> 7385	14=> 7386	15=> 7387	16=> 7391	17=> 7415	18=> 7417	19=> 7423	20=> 7424		
C	21=> 7392	22=> 7393	23=> 7412	24=> 7413	25=> 7422	26=> 7425	27=> 7426	28=> 7429	29=> 7431	30=> 7432		
D												
E (neg.)	FMO4 no RoRgt	FMO5 no Tbet	FMO6 no GATA3	FMO7 no FoxP3								
F (pos.)	FMO1 no RoRgt	FMO2 no Tbet	FMO3 no GATA3	FMO4 no FoxP3								
G												
H	Unst	Helios (R)	RoRgt (R)	Tbet (M)	CD25 (M)	GATA3 (R)	FoxP3 (R)	CD4 (M)				

Figure 6: Plate arrangement for FACS-Staining

Flowcytometry analysis The samples were measured using FACS-Canto II with the FACS-Diva software following the Protocol "LUMC 022.01: Standard Operating Procedure measuring samples manually with FACS-Canto" written by the Department of Parasitology at LUMC. The protocol was last reviewed by Kit Yeng Liu in May 2010. Before measuring the samples the machine was rinsed and the fluidic start-up was preformed. Then the parameters and channels were set and width and voltages adjusted. The unstained and single cell samples were run first and according to those measurements compensation was performed. Now the samples were acquired one by one and saved under the sample ID.

After the measurements it was noticed, that the data for the GATA3$^+$ cells was not usable, as the FACS machine was set up incorrectly for this specific readout. It was set up to measure channels FL1, FL2, FL3, FL4, FL6, FL7 and FL8, but not channel FL5. The original idea was to detect GATA3 staining in the FL6 channel, but this was the wrong detector as the used marker eFluor660 has to be detected using the FL5 channel. Therefore the GATA3 fluorescence could not be measured and the fluorescence recorded in FL6 was just background fluorescence and not due to GATA3. Thus no data about GATA3 was available.

FlowJo analysis The data gathered from FACS was analysed using FlowJo version 9. For this, the data was imported into the FlowJo software. The gating was performed as described below on one sample and then applied to all the other samples to obtain a standardized result. If the applied gate clearly did not match with the sample it was adjusted. For the selection of the gate, the single stainings and FMOs were taken in account.

Gating strategy for T cells The cell populations to be identified using FlowJo were the T helper cells (classified as the CD4$^+$ population). From these cells it was differentiated further in T regulatory cells (classified as CD4$^+$, CD25^{high} and FOXP3$^+$ cells), T helper 1 cells (classified as CD4$^+$ and T-bet$^+$ while being RORγt$^-$) and T helper 17 cells (classified as being CD4$^+$ and RORγt$^+$, while being T-bet$^-$). The CD4$^+$ population was given as a percentage of the total lymphocyte population, while all the other values were given as a percentage of the CD4$^+$ population.

The first gate set was the lymphocyte gate. For this the Forward Scatter Area (FSC-A) was plotted against the Sidewards Scatter Area (SSC-A) and the area where the lymphocytes are expected due to their size and granularity

was selected. After selecting the lymphocytes, the lymphocyte doublets were excluded by plotting once FSC-A against Forward Scatter Width (FSC-W) and SSC-A against Sidewards Scatter Width (SSC-W). To find the $CD4^+$ population FSC-A was plotted versus the CD4 marker and the cloud with CD4 expression was selected. For further gating only the $CD4^+$ cells were used. To find the T regulatory population CD25 was plotted against FOXP3 defining the T-regulatory cells as $CD25^{high}FOXP3^+$ cells. To get the T_h1 and T_h17 populations first the population being negative for the other marker was selected and then plotted versus the marker searched for. A FlowJo gating example for T cells is given in Figure 7.

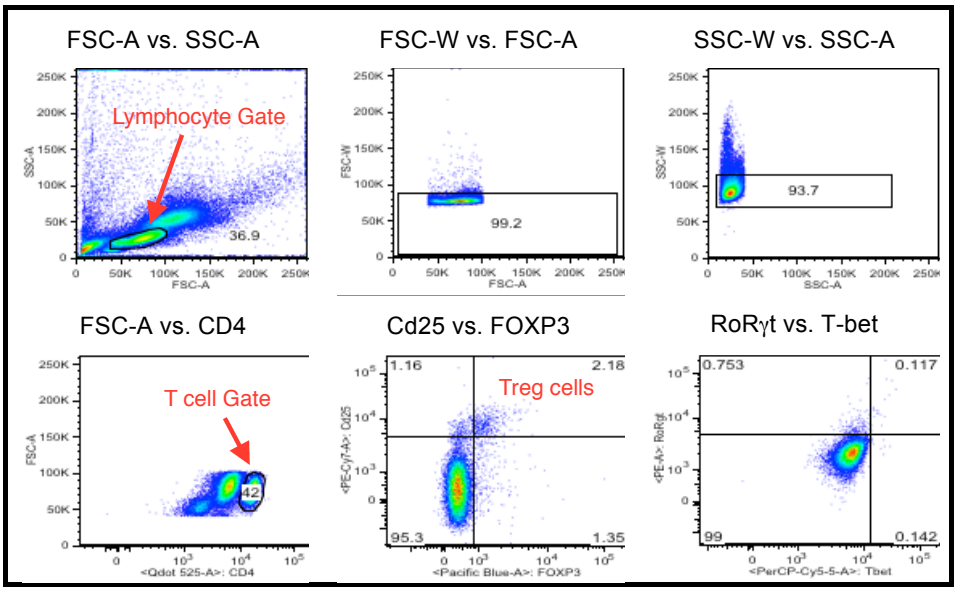

Figure 7: Gating example FlowJo

2.2.5 Data management and analysis

Collection of demographic data A questionnaire was completed for each woman included. In the first section information about the mother comprising age, area of habitation and number of former pregnancies and deliveries was collected. The second section was about the current pregnancy including date of last menstruation, diseases/complications during pregnancy and potential medications received. The last section contained information about the newborn comprising gestational age at delivery, sex, birth weight, length and head circumference. The collected data were entered into an openClinica data base

and included in the analysis later on to exclude other factors influencing the results.

Statistical Analysis All the data was analyzed using IBM SPSS Statistics Version 20. For this a SPSS data base was generated including the data gained from the questionnaire and the data generated from FlowJo.

In case the data was normally distributed an independent sample t-test was performed to compare continuous variables (for example percentage of T cell subsets) between the groups (for example filaria infected and uninfected or malaria history in pregnancy and no malaria history). In case the data were not normally distributed they were log transformed in order to get a distribution closer to normality. Data that remained not normally distributed were compared using a non parametric Man Whitney test. The same principle was applied to compare the study groups. Moreover the chi-squared test was used for comparing proportions.

The correlation between two continuous variables was assessed using the Spearman's correlation test. A positive Pearson coefficient (r) indicated a positive correlation between the two variables, while a negative Pearson coefficient indicated a negative correlation.

Since *P. falciparum* infection during pregnancy has the potential to modulate the immune system of the mother and could influence the one of the fetus we have decided to correct for effect of filaria on the newborn immune response after correcting for past episode of *P. falciparum* infection during pregnancy [78, 79, 80]. A multiple regression analysis was performed to see the independent effect of filaria and malaria infection on the different T cell subsets. The association of the two variables is given by the regression coefficient beta (β) (+β= positve association, -β= negative association).

A linear regression analysis was performed to asses the association between regulatory cells and the T_h1 and T_h17, first regardless of the filaria infection status of the mother and secondary in newborns from filaria infected and uninfected mothers separately. The strength of the association was determined by the level of the regression coefficient β.

The level of significance was set at a p value below 0.05.

2.2.6 Ethics

Ethical Approval In April 2010, the International Ethical Commission of the Albert Schweitzer Hospital in Lambaréné ("Comité d'Ethique Régional Indépendant de Lambaréné" (CERIL)) approved the study. The study was conducted in compliance to the study protocol and the participants' consent was obtained according to the ethical principles stated in the Declaration of Helsinki (stated by the World Medical Association at their 59th assembly in October 2008 in Seoul). The applicable guidelines of "International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use" (ICH), of Good Clinical Practice (GCP), and the applicable regulations of Gabon were followed. A copy of the signed and dated informed consent was given to the participants. The signed and dated original was retained with the study records.

Informed Consent A signed enrollment informed consent (or thumb-printed when the woman was illiterate) was obtained for the MiPPAD-Study before any tests related to the study were carried out. Additionally an ancillary study informed consent was signed by the women accepting to participate in this study. If the woman was under 18, additionally, the signature of a legal guardian was obtained. The informed consent covered the woman and the newborn.

2.3 Materials

2.3.1 Apparatus

Table 3: Apparatus list

Apparatus name	**Manufacturer**
Airstream® Class II Biological Safety Cabinet	ESCO, Singapore
Centrifuge Rotanta 460R/460RS	Hettich Zentrifugen, Germany
FACS Canto	BD Biosciences, US
ABX Pentra 60	HORBIA Medical, Japan
Water Still 2012	GFL®, Gesellschaft für Labortechnik mbH, Germany
Precision balance 440	KERN, Germany
Roller Tubes Mixer	Healthcares Technologies, South Africa
Pipetboy acu	Integra Biosciences, Switzerland
Pipette 2-20μl Labmate; LM20	Abimed, Germany
Pipette 20-200 μl; Labmate; LM200	Abimed, Germany
Pipette 100-1000 μl; Labmate; LM1000	Abimed, Germany
Neubauer improved-Counting chamber	LaborOptik, Germany
Refrigerator, 0 ºC	
Freezer, -80 ºC	

2.3.2 Materials

Table 4: Material list

Material	Manufacturer
Immunological Examinations	
Falcon® 50 ml conic tube	Becton Dickinson Labware, US
EasyLoad® universal 1000μl	Greiner Bio-one, Germany
EasyLoad® universal 200μl	Greiner Bio-one, Germany
10 ml Stripette Costar®	Corning Incorporated, US
Tranferpipette 3.5ml	SARSTEDT, Germany
CryoTube™Vials 1.8ml	Nunc™A/S, Denmark
Cryo-Babies®	Roth, Germany
96-well plate Round, Nunclon™Surface	Nunc™A/S, Denmark
FACS V-bottom F96 wells plate, non-sterile	Nunc™A/S, Denmark
1.4 ml FACS insert tubes	Micronic, Netherlands
FACS tubes	BD-Bioscience, US
Reaction tubes, 1.5ml	Greiner bio-one, Germany
1ml Sub-Q	Becton Dickinson, France
Parasitological Examinations *	
15ml Cellstar® Tubes	Greiner Bio-one, Germany
Microscope slide 76x26 mm ISO 8037/1	Langenbrinck, Germany
Cover slide 24x60 mm Dicke 0.13-0.18mm	Langenbrinck, Germany
Plastic Swinney filter holders	STERLITECH Corporations, UK
Polycarbonate screen membrane filters -13 mm diameter, 12 μm pore size	STERLITECH Corporations, UK
10 ml one time use syringes	HA SA MED, Germany
Staining Jar	
Blood sampling	
BD Vacutainer® Safety-Lok™21G	Becton Dickinson, US
Multi-Adapter	SARSTEDT, Germany
S-Monovette® EDTA K/2,7 ml	SARSTEDT, Germany
S-Monovette® Li-Heparin NH4/ 5,5 ml	SARSTEDT, Germany
S-Monovette® Li-Heparin NH4/ 9 ml	SARSTEDT, Germany
RAPI SWAP 70% Isopropyl Alcohol	Jagat Diagnostics Pvt. Ltd. India
One-time-use gloves Latex	Cooper Cooperation, France
Tourniquet	
Cotton	

2.3.3 Chemicals

Table 5: Chemical list - 1

Chemical	**Manufacturer**
CBMC Isolation	
Ficoll-Amidotrizoaat (Ficoll)	Apotheek AZL, Netherlands
Hanks' Balanced Salt Solution (HBSS)	GIBCO®, Invitrogen™, US
Iscove's Modified Dulbecco's Medium (IMDM)	GIBCO®, Invitrogen™, US
Sodium-Penicillin G 100U/ml	Astellas Pharma, Netherlands
Streptomycin 100μg/ml	Sigma-Aldrich, Netherlands
Glutamate (2mM)/Pyruvate (1mM)	Sigma-Aldrich, Netherlands
Fetal Bovine Serum (FCS)	Greiner Bio-one, Austria
Tuerk-stain	
eBioscience Fixation	
Phosphate Buffered Saline (PBS) 10x	Fresenius Kabi, Germany
eBio fixation/Permeabilization Diluent	eBioscience, Canada
eBio fixation/Permeabilization Concentrate	eBioscience, Canada
Dimethylsulfoxide (DMSO)	MERCK, Germany
Parasitological Examinations *	
Saponin powder	Sigma, US
Tuerk-stain	Merck, Germany
Giemsa-stain	
Distilled water	

Table 6: Chemical list - 2

Chemical	**Manufacturer**
FACS-Staining	
Compensation Bead Anti-Rat/Hamster Ig	BD-Bioscience, US
Compensation Bead Anti-Mouse Ig	BD-Bioscience, US
FcγR-binding inhibitor	eBioscience, Canada
Ethylenediaminetetraacetic acid (EDTA, 2mM)	Sigma-Aldrich, US
Anti-RORγt, PE, Rat IgG2a	eBioscience, Canada
Anti-T-bet, PerP5.5, Mouse IgG1	eBioscience, Canada
Anti-CD25, PE-Cy7, Mouse IgG1κ	BD-Bioscience, US
Anti-GATA3, Eflour660 Rat IgG2bκ	eBioscience, Canada
Anti-FOXP3, Eflour450, Rat IgG2aκ	eBioscience, Canada
Anti-CD4, V500, Mouse IgG1κ	BD-Bioscience, US

* Parasitological examinations for *L. loa, M. perstans, P. falciparum and Schistosoma haematobium*.

3 Results

3.1 Characteristics of the study population

A total of 30 pregnant women and their newborns were included in this study, of whom 15 were infected with either *L. loa* or *M. perstans* and 15 were free of these infections.

Considering the demographic characteristics both study groups were found to be comparable at inclusion (Table 7). The proportion of primiparus mothers was significantly higher in the uninfected group (0% in the infected group vs. 4% in the uninfected one, p= 0.032). A similar higher proportion of male newborns were born in the uninfected group (53% in the infected group vs. 87% in the uninfected one, p= 0.046). The hemogram from the mothers showed that filaria-infected mothers had significantly higher platelet counts (251x10^3/mm^3 in infected vs. 172x10^3/mm^3 in uninfected, p= 0.020) then the uninfected mothers. Also a trend for lower Hematocrit (34.5% in infected vs. 38% in uninfected, p=0.056) and Hemoglobin levels (11.08g/dl in infected vs. 12.27g/dl in uninfected, p=0.052) was seen in the infected group. All of the mothers were free of malaria at the time of delivery and two were found with *S. haematobium* infection (one in each group).

Table 7: Comparison of study groups

	Characterization Study Population		Filaria infected	Filaria uninfected	p value
Characteristics of the mothers	**Malaria episodes during pregnancy,** N (%)		8 (53%)	5 (33%)	0.269#
	S. haematobium infections, N (%)		1 (7%)	1 (7%)	1#
	Age of mother at delivery in years: Mean (±SD)		24.27 (±7.54)	25.47 (±5.44)	0.621*
	Area	**Fougamou,** N (%)	7 (47%)	5 (33%)	0.456#
		Outside, N (%)	8 (53%)	10 (67%)	
	Erythrocytes (x10^6/mm^3): Mean (±SD)		4.29 (± 0.52)	4.47 (± 0.99)	0.550*
	Leukocytes (x10^3/mm^3): Mean (±SD)		7.93 (± 3.13)	8.87 (± 2.67)	0.387*
	Hematocrit (%): Mean (±SD)		34.49 (± 4.22)	38.00 (± 5.35)	0.056*
	Hemoglobin (g/dl): Mean (±SD)		11.08 (± 1.48)	12.27 (± 1.71)	0.052*
	Platelets (x10^3/mm^3): Mean (±SD)		251.20 (± 99.98)	172.73 (± 71.08)	**0.020***
	Lymphocytes (x10^3/mm^3): Mean (±SD)		2.91 (± 1.00)	2.34 (± 0.81)	0.099*
	Eosinophils (x10^3/mm^3): Mean (±SD)		0.72 (± 0.70)	0.85 (± 0.49)	0.555*
	Basophils(x10^3/mm^3): Mean (±SD)		0.05 (± 0.03)	0.05 (± 0.02)	0.830*
	Monocytes (x10^3/mm^3): Mean (±SD)		0.49 (± 0.40)	0.44 (± 0.17)	0.686*
	Neutrophils (x10^3/mm^3): Mean (±SD)		3.76 (± 2.83)	4.97 (± 2.57)	0.237*
Characteristics of the child	**Mean gestational age in months**: Mean (±SD)		37.24 (± 3.26)	37.80 (± 3.75)	0.666*
	Mean birth weight in g: Mean (±SD)		2843.33 (± 413.81)	2950.67 (± 455.47)	0.505*
	Mean length at birth in cm: Mean (±SD)		47.00 (± 2.00)	48.00 (± 1.65)	0.147*
	Mean head circumference in cm: Mean (±SD)		31.60 (± 1.30)	32.33 (± 1.11)	0.108*
	Sex of child	**Male,** N (%)	8 (53%)	13 (87%)	**0.046#**
		Female, N (%)	7 (47%)	2 (13%)	

The categorical data was analyzed using the Pearson Chi Square test (marked #) while the continuous data was compared using an independent sample t-test (marked *).

3.2 Effect of demographic factors on the distribution of T cell subsets

The independent sample t-test was used to evaluate the effect of the sex of child and area of the mother living place, on the distribution of $CD4^+$ cells and $CD4^+$ cells expressing FOXP3 (T_{reg}), Tbet (T_h1) and RorγT (T_h17) (Table 8).

No differences were seen in percentages of $CD4^+$ cells, regulatory T cells, T_h1 and T_h17 cells between male and female children or considering the mothers living place (Table 8). Comparing T cell subsets of the female children to those of the male a trend for lower T_h1 cells (0.01% in female vs. 0.17% in male, p=0.052) was seen.

The effect of the influencing factors: maternal age, gestational age and birth weight on the distribution of $CD4^+$ cells and $CD4^+$ cells expressing FOXP3, Tbet and RorγT was analyzed using a correlation analysis (Table 8).

This analysis showed that the gestational age of the child had a significant influence on the regulatory T cells and T helper subsets. Gestational age showed a negative association with the regulatory T cells (Pearsons r= -0.477, p=0.008) and a positive correlation with T_h1 cells (Pearsons r=0.375, p=0.041) and T_h17 cells (Pearsons r=0.437, p=0.016). The age of the mother and birth weight of the child had no significant influence on the T cell subsets. There was a trend for a negative correlation between maternal age and regulatory T cells (Pearsons r=-0.322, p=0.083) and a trend for a positive correlation between maternal age and T_h1 cells (Pearsons r=0.329, p=0.076).

Table 8: The distribution of T cell subsets of newborns in relation with demographic factors and the infection status of their mother

		CD4+ T cells		T reg cells		Th1 cells		Th17 cells	
Factor	**Categories**	**Mean (± SD)**	**P**	**Mean (± SD)**	**P**	**Mean (± SD)**	**P**	**Mean (± SD)**	**P**
Filaria	Negative (15)	43.87 (± 2.88)	0.344	2.34 (± 0.19)	0.210	0.14 (± 1.15)	0.564	0.16 (± 1.17)	0.113
	Positive (15)	47.71 (± 2.76)		2.79 (± 0.30)		0.16 (± 1.26)		0.10 (± 1.20)	
Malaria in pregnancy	No (17)	46.82 (± 2.90)	0.561	2.26 (± 0.21)	0.048	0.17 (± 1.20)	0.246	0.13 (± 1.17)	0.732
	Yes(13)	44.43 (± 2.68)		2.97 (± 0.29)		0.12 (± 1.17)		0.12 (± 1.26)	
Sex of child	Female (9)	44.62 (± 4.93)	0.709	2.52 (± 0.38)	0.871	0.1 (± 1.20)	0.052	0.14 (± 1.29)	0.718
	Male (21)	46.29 (± 2.00)		2.59 (± 0.21)		0.17 (± 1.17)		0.12 (± 1.17)	
Area	Fougamou (12)	45.98 (± 3.21)	0.940	2.49 (± 0.28)	0.730	0.14 (± 1.20)	0.674	0.12 (± 1.20)	0.785
	Outside (18)	45.66 (± 2.62)		2.62 (± 0.24)		0.15 (± 1.20)		0.13 (± 1.20)	

Independent sample t test - The means in each group $\pm$ the standard deviation are given. p values are given to indicate the level of significance.

	CD4+ T cells		T reg cells		Th1 cells		Th17 cells	
Factor	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Age of mother	0.138	0.468	-0.322	0.083	0.329	0.076	0.264	0.159
Gestational age of the child	-0.477	0.008	-0.323	0.081	0.375	0.041	0.437	0.016
Birth weight of the child	0.071	0.709	-0.039	0.838	-0.059	0.758	0.156	0.411

Correlation analysis - The strength of the correlation between two variables is given by the value of the Pearson coefficient (r) value. A positive r-value indicates a positive correlation between the variables, while a negative r-value indicates a negative correlation. p values are given to indicate the statistical significance of the correlation.

3.3 Effect of filaria infection on the different T cell subsets

The independent sample t-test was used to evaluate the difference in mean percentages of the filaria infected and uninfected group in $CD4^+$ cells and $CD4^+$ cells expressing FOXP3, Tbet and RorγT (Table 8).

Between the filaria infected and uninfected groups no significant difference was found in percentage of $CD4^+$ cells (47.71% in infected vs. 43.87% in uninfected, p=0.344), T_{reg} (2.79% in infected vs. 2.34% in uninfected, p=0.210), T_h1 cells (0.16% in infected vs. 0.14% in uninfected, p=0.564) and T_h17 cells (0.10% in infected vs. 0.16% in uninfected, p=0.113).

As several studies have shown that maternal malaria infection during pregnancy can effect the fetal immune response including the T cells [78, 79, 80], a second analysis was performed to correct the outcome for episodes of malaria during pregnancy. This multiple regression analysis (Table 9) shows the independent effect of filaria infection on the distribution of percentages of $CD4^+$ cells and $CD4^+$ cells expressing FOXP3, Tbet and RorγT.

Again filaria infection showed no significant effect on the percentages of $CD4^+$ cells (β=3.504, p=0.404) and $CD4^+$ cells expressing FOXP3 (β=0.625, p=0.073), Tbet (β=0.060, p=0.334) and RorγT (β=-0.055, p=0.140).

3.4 Effect of malaria episode during pregnancy on the distribution of T cell subsets

The independent sample t-test was used to evaluate the effect of episodes of malaria during pregnancy on the mean percentages of $CD4^+$ cells and $CD4^+$ cells expressing FOXP3, Tbet and RorγT (Table 8).

Children born from mothers who experienced an episode of malaria during pregnancy had a significantly higher percentage of regulatory T cells in comparison to those who did not experience an episode of malaria during their pregnancy (2.97% in the group with malaria episode vs. 2.26% in the group without malaria episode, p= 0.048). No significant difference was seen between the means of $CD4^+$ cells (44.43% in the group with malaria episode vs. 46.82% in the group without malaria episode, p=0.561), T_h1 cells (0.12% in the group with malaria episode vs. 0.17% in the group without malaria episode, p=0.246) and T_h17 cells (0.12% in the group with malaria episode vs. 0.13% in the group without malaria episode, p=0.732).

In other studies an effect of filaria infection status on the T cells was shown

[2, 6, 63]. Therefore as done for filaria infection the outcomes for history of malaria in pregnancy were corrected for filaria infection status using the multiple regression analysis. This shows the independent effect of episode of malaria during pregnancy on the distribution of percentages of $CD4^+$ cells and $CD4^+$ cells expressing FOXP3, Tbet and RorγT (Table 9).

In women with a malaria episode during pregnancy the regulatory T cells (β=0.839, p=0.019) were significantly higher then in those without an malaria episode. No difference was observed comparing the $CD4^+$ cells (β=3.504, p=0.404) and $CD4^+$ cells expressing Tbet (β=0.060, p=0.334) and RorγT (β=-0.055, p=0.140).

Table 9: Independent effect of filaria and malaria infection status of the mother on the distribution of T cell subsets in their newborns

			CD4+ T cells		T reg cells		Th1 cells		Th17 cells	
Factor	**Baseline**	**Category**	**β (95% CI)**	**P**	**β (95% CI)**	**P**	**β (95% CI)**	**P**	**β (95% CI)**	**P**
Filaria	Negative (15)	Positive (15)	3.504 (-4.99 to 11.99)	0.404	0.625 (-0.06 to 1.31)	0.073	0.060 (-0.066 to 0.186)	0.334	-0.055 (-0.13 to 0.02)	0.140
Malaria in pregnancy	No (17)	Yes (13)	-1.676 (-10.25 to 6.89)	0.691	0.839 (0.15 to 1.53)	0.019	-0.075 (-0.202 to 0.052)	0.236	-0.013 (-0.09 to 0.06)	0.718

The strength of the association between two variables is given by the value of the regression coefficient beta (β) value. A positive β-value indicates a positive association between the variables, while a negative β-value indicates a negative association. p values are given to indicate the statistical significance of the associations.

3.5 Association between regulatory T cells and T_h1 and T_h17 cell subsets in children born from filaria infected and uninfected mother

In order to investigate the association between regulatory T cells and the different T helper subsets, the association between $CD4^+CD25^{high}FOXP3^+$ and $CD4^+Tbet^+$, $CD4^+ROR\gamma T^+$ cells was examined through a linear regression analysis (Figure 8). Analyzing all subsets together a negative association

between regulatory T cells and $CD4^+Tbet^+$ T cells (β=-0.149, 95% CI= -0.256 to -0.043, p=0.008) or $CD4^+ROR\gamma T^+$ T cells (β=-0.175, 95% CI= -0.275 to -0.074, p=0.001) was observed. After stratifying our study subjects by their filaria infectious status this negative association between the regulatory T cells and T_h1 (β=-0.242, p=0.002) or T_h17 (β=-0.170, p=0.013) was only observed in the filaria infected group. In the filaria uninfected group no significant negative association was seen between regulatory T cells and T_h1 cells (β=0.012, p=0.888) and T_h17 cells (β=-0.142, p=0.177).

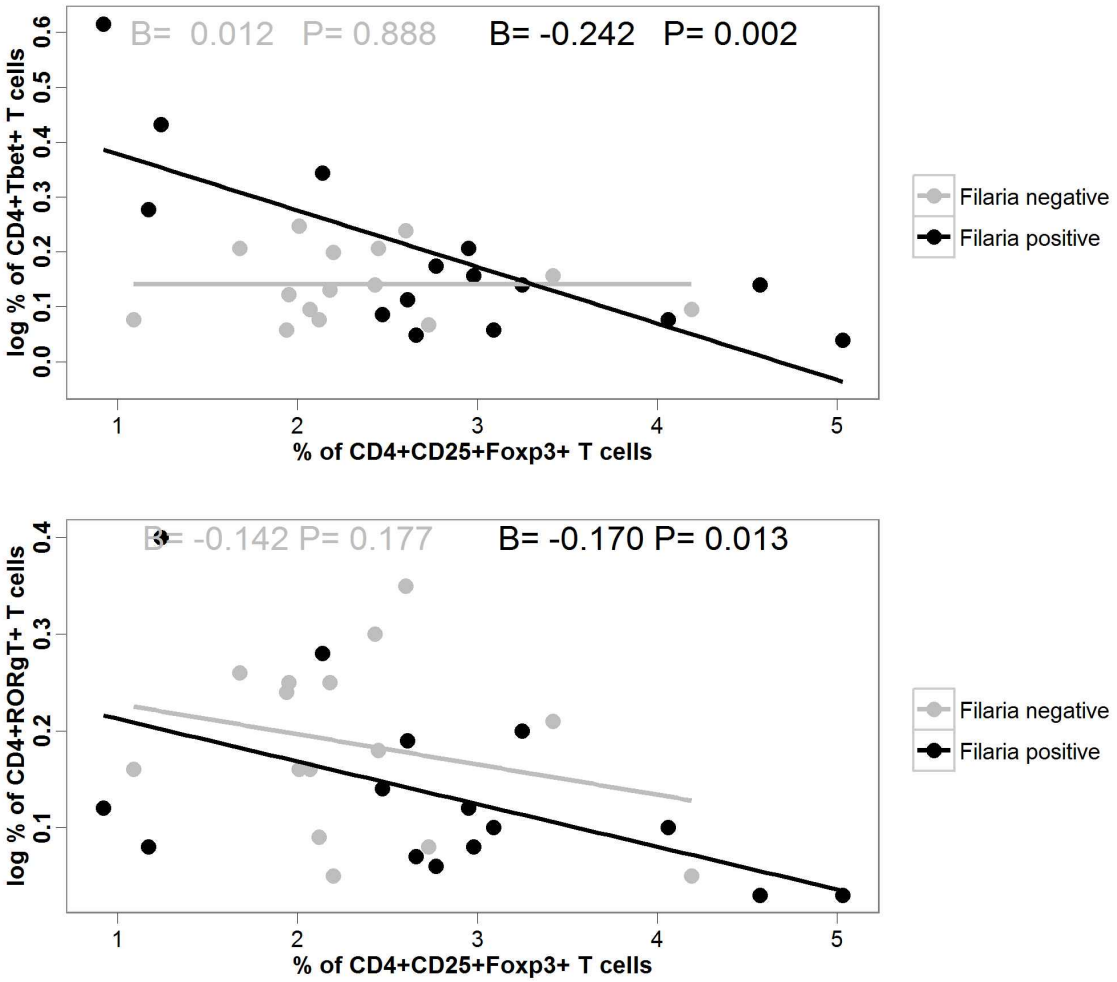

Figure 8: Association between regulatory T cells and T_h1 and T_h17 cell subsets in children born from filaria infected and uninfected mother

The relation between $CD4^+CD25^{high}FOXP3^+$ cells and $CD4^+Tbet^+$ cells (upper panel) as well as $CD4^+CD25^{high}FOXP3^+$ cells and $CD4^+ROR\gamma T^+$ cells (lower panel) of CBMC of neonates from filaria negative (in grey) and filaria positive (in black) mothers assessed by a linear regression analysis. Each dot shows a single subject while the solid lines represent the regression lines of the model. The strength of the association between two variables is given by the value of the regression coefficient beta (β represented by B in our graph) value in each graph. A positive β value indicates a positive association between the variables in the model, while a negative β value indicates a negative association. p values are given to indicate the statistical significance of the associations.

4 Discussion

This exploratory study was designed to investigate the effect of in utero exposure to filarial antigen on the expression of T helper cell populations in cord blood. For this the percentages of $CD4^+$ T cells and $CD4^+$ T cells expressing Tbet, RORγT and FOXP3 were measured in CBMC from filaria infected and uninfected mothers.

Helminths including filariasis are very common infections in the developing world [81, 9, 10]. Their ability to chronically infect their human host for decades is linked to their strong immune down regulatory function. Parts of this down regulatory function are caused by $CD4^+$ T cells. By producing cytokines and co-stimulatory signals $CD4^+$ T cells are able to influence the function and responsiveness of the immune system to different antigens. Especially the regulatory T cells are of great importance for this immune modulation [5]. Previous studies have shown that chronic filaria infection in adults induces regulatory T cells and alternatively activated macrophages, which then lead to a suppression of the T_h1 and T_h2 cells [2, 61]. Increasing evidence suggests that this down-regulatory effect does not just apply to adults, but during pregnancy can even extend to the child. Children born to filaria-infected mothers seem to have increased susceptibility to filarial infection leading to earlier infection in life, higher parasite loads and fewer disease manifestations [2, 3, 4]. Several studies from different areas indicate that children born to filaria infected mothers display a biased immune response to filaria antigens, with higher levels of the down regulatory IL-10 and IgG4 as well as a reduced T lymphocyte responsiveness [2, 63, 82, 83, 84]. In our study no significant effect of filaria infection status on the percentages of $CD4^+$ T cells, regulatory T cells, T_h1 cells and T_h17 cells was found. Other studies reported higher levels of IL-10 which would go in line with increased T_{reg} cells [6, 85]. We were not able to reproduce those results, which might be due to the limiting factors reported below, like the possibility of other geohelminth infections of the mother or the low specificity/sensitivity of the Leuko-concentration method used to detect the mf. A study in Kenya by Malhotra et al. found that maternal *Wucheria Bancrofti* infection leads to an increase in IL-5 skewing the child's immune response towards T_h2, while no differences were found for the levels of the T_h1 cytokines IL-2 and IFN-γ [3]. While another study by Steel et al. comparing Polynesian adolescents born to microfilaremic and uninfected mothers showed that the infected group had lower levels of the T_h1 cytokines IL-2, IL-5 and IFNγ [84]. These finding compare with our result seeing no increase in T_h1 cells and the negative correlation between T_{reg} and T_h1 cells in the infected group.

Unfortunately we were not able to reproduce the result of a possible increase in T_h2 cells as the respective data was not usable (see Material and Methods, Flowcytometry analysis).

Continuing our analysis looking at the association between the regulatory T cells and T_h1 and T_h17 cells a negative correlation of T_{reg} with Tbet and RORγT cells was found to be significant only in the infected group. This correlation was not found in the filaria uninfected group and might be explained by a stronger functional activity of the regulatory T cells in infected individuals. This finding compares to a study by Wammes et al. [86], where frequencies and function of $CD4^+CD25^{high}FOXP3^+$ regulatory T cells in geohelminth infected and uninfected induviduals were compared. This study described that while the frequencies of regulatory T cells between the two groups were similar their suppressive activity was more pronounced in the geohelminth infected group [86]. The same result was found for lymphatic filariasis [46]. This in combination with our data might suggest that an activation of $CD4^+CD25^{high}FOXP3^+$ regulatory T cells occurs upon exposure of the cell to parasite antigens leaving them with strong functional capacity.

Similar to filaria infection previous studies have shown that infants born to malaria infected mothers have a higher post natal risk for an acute malaria episode and also show a higher prevalence for blood parasites [87, 88]. In this context several studies investigated the effect of malaria during pregnancy on the immune system. Our findings that regulatory T cells were increased in cord blood from mothers with at least one episode of malaria during pregnancy went in line with findings from other studies. A study by Malhotra et al. [78] showed that children from mothers with placental malaria demonstrated high levels of IL-10 in response to in-vitro stimulation. Three other studies using in-vitro depletion were able to demonstrate the involvement of regulatory T cells in this process, leading to a restriction of the T_h1 cytokine IFNγ [78, 79, 80]. In all those studies the mothers presented with either placental malaria or were malaria positive at birth. In our study all mothers were free of malaria infection at the time of birth showing that this immune priming effect of malaria infection can already take place in earlier pregnancy. Furthermore we were not able to observe a decrease in T_h1 percentages like in the studies mentioned before. This might be due to the fact that the mothers in the malaria infected group were both primi and multi parus. A study published by Malhotra et al. [89], which was looking at the effect of placental malaria on T cell population in babies described that babies born to first time mothers present with a T_h1 or a mixed T_h1 and T_h2 response, while babies from

multiparus mothers show a T_h2 response to these parasite antigen [89].

At the time of birth the immune system has not completely matured and full maturation is only reached in the first childhood years. Therefore the immune system of term infants shows a reduced innate as well as adaptive immunity. The status of preterm birth (< 37 weeks of gestation) can increases this effect. Preterm children show lower production of cytokines which limits T cell activation [58]. In our study we were able to show a significant negative correlation of gestational age and $CD4^+$ T cells. This finding has been reported in a study by Series et al. where preterm infants showed higher levels of $CD4^+$ cells compared to term infants [90]. Another study published in 2014 demonstrated a negative correlation between gestational age and regulatory T cells [91]. Even though this correlation was not significant in our analysis a trend for a negative correlation was observed. The positive correlation of gestational age and T_h1 as well as T_h17 subsets might be explained by the T_h2 bias during pregnancy. During fetal life the fetal cytokine response is driven toward T_h2 restricting the pro-inflammatory T_h1 cells. This bias is thought to be pretentious of fetal rejection by the mothers immune system [58]. With increasing gestational age the bias might decrease leading to higher numbers of T_h1 as well as T_h17 cells as shown in our study.

There were some limitations to this study the most obvious being the small sample size that might have prevented the detection of significant differences in percentage of $CD4^+$, T_h1, T_h17 and T_{reg} populations. Also the not usable data for the T_h2 cells leaves questions open considering the extend of their involvement in the immune response.

Another factor that we did not consider to the necessary extend was the bias of co-infections like intestinal helminths and other parasite infections. Knowing that all infections experienced during pregnancy may have an effect on the child's immune response those possible infections might have influenced our data and influenced the levels of significance. But considering the high prevalence of these parasitic infections in the study area, their distribution in the study population was assumed to be homogenous. Additionally to those limiting factors the question has to be raised whether the leuko-concentration method is a suitable enough test to determine the filaria infection status, as it only is positive when patients present with mf in blood. Due possibility of amicrofilaremic infection there is a chance of not finding mf even though the mother was infected. This may have lead to filaria infected subjects hiding in the uninfected group leading to a decrease in significance. The analysis for specific IgE or IgG in cord blood to determine whether the newborn was exposed might be a suitable possibility to overcome

this limitation.

Nevertheless this thesis was able to show that offspring born to filaria infected mothers have regulatory T cells that are negatively associated with T_h1 and T_h17 cells. These alterations caused by maternal helminth infection may lead to a poor immunological response to future antigens encountered and vaccinations administered during the first years of life and thus leaving the children at higher risk for childhood infections.

In the future it might be interesting to consider whether women in the childbearing age should be screened and treated for *L. loa* and *M. perstans* before a possible pregnancy. This would allow the child's immune response to develop without the down regulatory limitations that could be detrimental for its health.

5 Summary

5.1 Summary English

There is increasing evidence that maternal infections during pregnancy can affect the developing immune system even if no vertical transmission is taking place. Examining filaria infection different studies have shown evidence that the immune deviation already starts in utero and children born to filaria-infected mothers seem to have increased susceptibility to filarial infection.

In this study 30 pregnant women were recruited, examined for their filaria infection status and divided into two homogenous groups, filaria infected and uninfected. At delivery, cord blood mononucleated cells were obtained and the $CD4^+$ cells were phenotyped by expression of the transcription factors Tbet, RoRγT and FOXP3.

No significant differenced in percentage of $CD4^+$ cells, regulatory cells, T_h1 and T_h17 cells were observed comparing the filaria infected with the uninfected group. However in the infected group only there was a negative association between $CD4^+CD25^{high}FOXP3^+$ T cells and $CD4^+Tbet^+$ T cells (B=-0.242, p=0.002) as well as $CD4^+ROR\gamma t^+$ T cells (B=-0.170, p=0.013).

Those results suggest that filaria infection during pregnancy leads to an expansion of functionally active regulatory T cells in the child that keep T_h1 and T_h17 cells under control. As a secondary outcome it was possible to reproduce a previous finding that malaria infection during pregnancy leads to an increase in regulatory T cells.

These alterations caused by maternal helminth and malaria infection may lead to a poor immunological response to future antigens encountered and vaccinations administered during the first years of life and thus leaving the children at higher risk for childhood infections.

5.2 Summary German

Nach heutigem Stand der Forschung gibt es zunehmende Hinweise dafür, dass Infektionen während der Schwangerschaft einen Einfluss auf das sich entwickelnde Immunsystem des Fötus haben. Dieser Effekt besteht unabhängig von einer vertikalen Übertragung des Erregers auf das Kind. Verschiedene Studien, welche sich mit der Infektion durch Filarien während der Schwangerschaft beschäftigt haben, beschreiben, dass der Einfluss auf das Immunsystem des Kindes schon im Mutterleib zu beobachten ist, und dass für Kinder von Filarien infizierten Müttern ein erhöhtes Risiko einer Filarieninfektion besteht.

Für die hier beschriebene Studie wurden 30 schwangere Frauen rekrutiert, auf ihren Filarieninfektionsstatus untersucht und in zwei homogene Gruppen unterteilt: infiziert und nicht infiziert. Bei der Geburt wurden die mononukleären Zellen aus dem Nabelschnurblut isoliert und die $CD4^+$ Zellen wurden auf die Expression der Transkriptionsfaktoren Tbet, RoRγT und FOXP3 hin analysiert.

Es wurden keine signifikanten Unterschiede in den Anteilen von $CD4^+$ Zellen, regulatorischen T Zellen , T_h1 und T_h17 Zellen zwischen den beiden Testgruppen beobachtet. In der Filarien infizierten Gruppe wurde jedoch eine negative Assoziation zwischen $CD4^+CD25^{high}FOXP3^+$ Zellen und $CD4^+Tbet^+$ T Zellen (B=-0.242, p=0.002) sowie $CD4^+ROR\gamma t^+$ T Zellen (B=-0.170, p=0.013) gefunden. Diese war in der nicht infizierten Gruppe nicht vorhanden. Diese Ergebnisse legen nahe, dass eine Filarieninfektion während der Schwangerschaft beim Kind zu einer vermehrten Aktivierung von regulatorischen T Zellen führt, welche die T_h1 und T_h17 Zellen unter Kontrolle halten.

Im Weiteren konnten durch die Studie frühere Ergebnisse reproduziert werden, welche zeigten, dass eine Malariainfektion während der Schwangerschaft zu einem Ansteigen der regulatorischen T Zellen führt.

Die Veränderungen des Immnsystems, welche durch eine Infektion mit Helminthen und Malariaerregern, verursacht werden, können während der ersten Lebensjahre des Kindes zu einer verminderten Immunantwort gegenüber neuen Antigenen bzw. Impfungen führen. Dies hat ein erhöhtes Infektionsrisiko für die betroffenen Kinder zur Folge.

6 Appendix

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References

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7 Erklärung zum Eigenanteil der Dissertationsschrift

DIe Konzeption der Studie erfolgte in Zusammenarbeit mit Prof. Dr. M. Yazdanbakhsh (Department of parasitology, LUMC), Dr. U. Ateba Ngoa (Department of parasitology, LUMC) and Dr. G. Mombo Ngoma (Centre de Recherches Médicales de Lambaréné).

Dr. U. Ateba Ngoa hat mir bei der Auswahl der statistischen Tests geholfen, die Arbeit betreut und Korrektur gelesen.

Frau L. van der Vulgt (Department of parasitology, LUMC) hat mich in die Arbeit im Labor eingelernt und die FACS-Messungen gemeinsam mit mir durchgeführt.

Die Patientenbehandlung und Entnahme des Nabelschnurblutes wurde von den Mitarbeitern der MiPPAD-Studie durchgeführt.

Die folgenden Arbeiten wurden von mir eigenständig durchgeführt:
– Rekrutierung, Blutentnahme, Filariendiagnostik und Datenerhebung
– Durchführung der Zellisolation und fixierung
– Auswertung und statistische Analysen

Ich versichere das Manuskript selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

8 Publikation

Ateba-Ngoa U, Mombo-Ngoma G, Zettlmeissl E, van der Vlugt LE, de Jong S, Matsiegui PB, Ramharter M, Kremsner PG, Yazdanbakhsh M and Adegnika AA. CD4+CD25hiFOXP3+ Cells in Cord Blood of Neonates Born from Filaria Infected Mother Are Negatively Associated with CD4+Tbet+ and CD4+RORγt+ T Cells. PLoS One. 2014 Dec 22;9(12):e114630. doi: 10.1371/journal.pone.0114630. eCollection 2014.

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