CHAPTER 2

Loa loa infection and the balance of Th17 and regulatory T cells

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Abstract

Background Filarial infections are associated with profound changes in the immune system. However, relatively little is known about *Loa loa* infection which causes subcutaneous filariasis and shows a spectrum of clinical manifestations.
Methods To characterize the effect of *Loa loa* infection on the immune system, the levels of IFN-γ, IL-5, IL-13, IL-10 and IL-17 produced by peripheral blood mononuclear cells in response to filarial antigen and mitogen were measured in infected subjects with and without microfilariae in blood as well as in endemic control subjects. Moreover the frequencies of CD4+ T cell subsets (regulatory T cells, Th1, Th2 and Th17 cells) were analyzed in the three groups using flow cytometry.
Results Levels of Th2-type cytokines were significantly higher in amicrofilaremic infected subjects whereas microfilaremics had lower levels of both Th1 and Th2 cytokines. The infected groups showed low levels of IL-17 and IL-17 producing cells while showing significantly higher regulatory T cells compared with endemic controls.
Conclusions These results suggest that *Loa loa* infection is associated with expansion of Th2 responses, however, when microfilaremic, the infection is associated with suppressed antigen specific Th2 as well as Th1 responses. *Loa loa* infections, irrespective of patent microfilaremia, lead to expansion of regulatory T cells and decrease in frequency of Th17 cells, which might prevent excessive inflammation in tissues affected by this parasite.

Introduction

Loa loa is a human filarial parasite, endemic in West and Central African rain forests[1,2]. In endemic areas a proportion of exposed subjects can remain uninfected. The clinical spectrum of loiasis ranges from asymptomatic infection to typical clinical symptoms such as Calabar swelling, pruritus and ocular passage of the adult worm. Occasionally more severe complications such as pulmonary abnormalities, renal failure, cardiomyopathy and encephalitis have been reported[3,4].

"Occult loiasis" is the term used for patients who have no microfilaria in peripheral blood despite evidence of infection as determined by clinical signs and / or ocular passage of adult worms. In highly endemic areas, occult loiasis has been reported to be the most common infection state[5,6]. Although the low sensitivity of the diagnostic method, single-worm or single-sex infections could explain the high proportion of amicrofilaremic patients, it is thought that immunological mechanisms play an important role in controlling levels of microfilariae[7]. The immunological patterns that may reflect the diversity of the clinical manifestations remain poorly understood in loiasis. Early studies comparing inhabitants of endemic areas with temporary residents showed that infection in temporary residents leads into increased levels of parasite specific IgG, elevated IgE, profound hypereosinophilia and increased filarial antigen-specific lymphocyte proliferative responses and raised $CD4^+/CD8^+$ ratios[4]. As in other filarial infections, IgG4 is associated with active *Loa loa* infection, in both microfilaremic and amicrofilaremic subjects[8]. Th2 cytokines IL-4, IL-5 and IL-13, which are known to be responsible for eosinophilia and IgE as well as IgG4 isotype switching[9,10], are enhanced in response to polyclonal stimulation in microfilaremic loiasis patients[11]. However, filarial antigen-specific IFN-γ, IL-2, IL-4 and IL-5 production by peripheral blood mononuclear cells (PBMC) and lymphocyte proliferation have been reported to be diminished in microfilaremic subjects[12]. Moreover, studies of experimental *Loa loa* infection in mandrills showed that appearance of microfilaria was associated with decreased proliferation of T cells and low levels of IFN-γ, IL-2, IL-4 and IL-5 production in response to filarial antigens[13]. These data indicate that loiasis leads to overall polarization of immune responses towards Th2 responses and actively suppresses antigen-specific responses, a feature that has already been described for other helminth infections[14]. The T cell hyporesponsiveness in filarial infection is thought to allow the long-term survival of the parasites within their host[14].

Although several studies have scrutinized Th1 and Th2 responses in parasitic filarial infections, there is relatively little data on regulatory T cells (Treg) or Th17 cells. Treg cells are a recognized subset of $CD4^+$ T cells that suppress effector cells. There is some evidence that Treg cells are involved in the downmodulation of

immunological responses to filarial infection, particularly in animal models. Infection of mice with *Brugia malayi* resulted in the expansion of $CD25^{hi}Foxp3^{+}$ T cells within the $CD4^{+}$ T cell population, accompanied with raised CD103 and CTLA-4 expression[15]. Babu and co-workers have shown in humans that infection with *Wuchereria bancrofti*, results in impaired induction of Tbet and GATA3 mRNA while the expression of FOXP3 and regulatory effectors such as TGF-β, and CTLA-4 are enhanced[16]. A fourth lineage of $CD4^{+}$ cells, the Th17 cell, has been described that appears to play an important role in pathogenesis of inflammatory diseases mediated by the signature cytokine IL-17[17]. A recent study in an area endemic for lymphatic filariasis in India has shown that in subjects with chronic lymphedema but with no microfilaria or circulating antigens, antigen specific Th1 and Th17 responses are elevated compared to individuals who are infected but asymptomatic. Moreover, lymphedema was associated with impaired expression of FOXP3, GITR, CTLA-4 and TGF-β mRNA[18]. However, the latter study did not examine the responses in uninfected subjects living in the same endemic area. In a study on lymphatic filariasis in Indonesia, IL-17 production was compared between MF-positive and -negative subjects and found to be lower in microfilaremics, but the lower response did not seem to be mediated by Treg cells[19]. Furthermore, asymptomatic amicrofilaremic individuals have recently been assessed in a *W. bancrofti*-endemic area in Ghana showing higher IL-17 responses to *B. malayi* antigen compared to asymptomatic MF-positive subjects [20]. These data suggest that Th17 responses are suppressed in MF-positive lymphatic filariasis patients and that if IL-17 suppression is lost it may lead to pathology.

In loiasis, which has a different clinical presentation than lymphatic filariasis, there are very few cellular immunological studies and none regarding the expression of Treg cells or IL-17 producing cells. The current study examines the effect of *Loa loa* infection on the cellular immune responses of individuals residing in an endemic area in Gabon.

Methods

Study population and hematological analysis

The study was carried out in Lambaréné, Gabon at the Albert Schweitzer Hospital. Lambaréné is an area highly endemic for *Loa loa* infection[11], located in dense rainforest. The study was approved by the *Comité d'Éthique Régional Indépendant de Lambaréné* (CERIL), Lambaréné, Gabon. Study subjects were mainly blood donors or relatives of children attending the outpatient clinic coming from the vicinity of the hospital and willing to participate in the study.

Inclusion criteria were (i) aged >18 years old, (ii) living in the study area for at least since 5 years prior to the study and (iii) written informed consent. Exclusion criteria were (i) pregnant and breastfeeding women (ii) treatment with anti-filarial drugs within the last 6 months prior to the study, (iii) presence of severe clinical conditions. All candidates fulfilling the criteria for enrolment were screened to determine their *Loa loa* infection status. Hematological analysis was performed with venous blood collected in EDTA tubes using ADVIA 120 Hematology System (Bayer HealthCare, Germany).

Detection of *Loa loa* and categorization of infection status

Detection of microfilaria

Three Giemsa-stained thick-blood smears from capillary blood were collected during three consecutive days. If all the three blood smears were negative for *Loa loa* microfilaria, 1.2 mL of venous EDTA blood was filtered (Nucleopore filtration), filters were stained with Giemsa and examined microscopically. Since *Loa loa* microfilariae exhibit a marked diurnal periodicity, blood samples were always obtained between 10:00 am and 2:00 pm. The blood thick smears were also checked for concomitant infection by *Plasmodium falciparum*, *Plasmodium malariae* and for the other filarial parasite *Mansonella perstans.*

Filaria IgG4 detection in serum by immunochromatography

Filaria-specific IgG4 detection in serum was performed by using an indirect immunochromatograhic assay (panLF rapid test, Reszon Diagnostics International Sdn. Bhd Subang Jaya, Malaysia) which contains BmR1 and BmSXP recombinant antigens that are cross-reactive with *Loa loa* [21].

History of Eye Worm
A specific questionnaire, based on the RAPLOA procedure [22], was administered by a medical doctor in order to assess eye worm passage, pathognomonic for *Loa loa* infection.

Categorization of Loa loa infection status
Study subjects were considered exposed to *Loa loa* infection, since all were residents of the same village. Subjects with microfilaria in at least one Giemsa-stained thick blood smear or positive on Nucleopore filter were included in the infected microfilaria-positive group (MF+). Subjects with history of eye worm passage during the 3 months prior to the study, negative thick blood smears or Nucleopore filters but detectable filaria-specific IgG4 by immunochromatography were considered infected microfilaria-negative participants (MF–). Endemic controls (EN) had no detectable filaria-specific IgG4, no microfilaremia and no history of eye worm passage.

Detection of geohelminths and *Schistosoma haematobium* infections
Stool samples were collected on two consecutive days and examined by the modified Kato-Katz method[23] for detection and quantification of *Ascaris lumbricoides* and *Trichuris trichiura* eggs. In addition, 7 days coproculture was performed and examined for the presence of hookworm eggs by microscopy. Urinary schistosomiasis was assessed by mid-morning terminal urine filtration and subsequent microscopy to detect *Schistosoma haematobium* eggs. Participants were considered infected if positive in at least one of three consecutively collected urine samples.

Blood sampling and PBMC culture
Blood samples for immunological analysis were taken by sterile venipuncture and collected into tubes containing sodium heparin. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll gradient centrifugation and suspended in culture medium (RPMI 1640 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2mM glutamine and with 10% heat-inactivated fetal calf serum, Gibco BRL). In total, 500.000 PBMCs were cultured at 37°C, 5% CO_2 in the presence of PHA (2 µg/mL) or *Brugia malayi* adult worm antigen (BmA, 12.5 µg/mL). BmA was prepared by homogenization of adult worms on ice in PBS containing 0.5% n-octyl glycoside (PBS-nOG). The homogenates were centrifuged, sterilized by filtration (0.45 µm filter), aliquotted and stored at – 80°C until use [21]. BmA is cross-reactive with *Loa loa* and has been previously used in patients infected with *Loa loa* to examine their immune responses[24]. After 72 hours culture with mitogen or antigen, supernatants were taken and stored at -20 °C until analysis.

Cytokine analysis in supernatants

Cytokine production in supernatants was assessed using the Multiplex Bead Immunoassay for interferon-gamma (IFN-γ) and interleukins (IL-5, -10, -13 and -17A) according to the manufacturer's instructions (Biosource, Invitrogen, Carlsbad, CA, USA) using Luminex 100™ xMAP technology (Luminex Corp., Austin, TX, USA). When cytokines in a sample were below the detection limit, a value corresponding to half the detection limit of the assay (given by manufacturer) was assigned to the sample.

Intracellular cytokines and circulating Tregs analyzed by flow cytometry

For analysis of intracellular cytokines 2×10^6 PBMCs were suspended in culture medium and transferred into 5 mL sterile tubes. Cells were stimulated with phorbol myristate acetate (PMA, 50 ng/ml) plus ionomycin (1 μM) for 2h and then an additional 4h in the presence of Brefeldin A (5 ng/ml). Cells were fixed with 1,9% formaldehyde (PFA), transferred into cryotubes and stored at -80°C. The fixed cells were thawed, permeabilized and stained with fluorochrome-labeled anti-CD3 (eBioscience Inc., San Diego, CA, USA), anti-CD4 (Invitrogen), anti-IFN-γ, anti-IL-4 (BD Biosciences, Franklin Lakes, NJ, USA), anti-IL-10 and anti-IL-17 antibodies. Stained cells were acquired on a BD LSRII flow cytometer (BD Biosciences).

To analyze regulatory T cells, 2×10^6 cells were fixed and permeabilized with a FOXP3 Staining kit (eBioscience). Afterwards, cells were washed with PBS, suspended in culture medium, transferred into cryotubes and stored at -80 °C. Cells were stained with fluorochrome-labeled anti-CD3 (eBioscience), anti-CD4 (Invitrogen), anti-CD25 (BD Biosciences) and anti-FOXP3 antibodies (eBioscience). and acquired using a FACSCanto analyzer equipped with FACSDiva software. Flow cytometry data were analyzed using FlowJo software (Treestar Inc., Ashland, USA).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism v.6 (Graph Pad Software Inc., San Diego). When data were not normally distributed, non-parametric analysis was performed. Differences between the three groups were analyzed first by ANOVA or Kruskal Wallis and if significant, differences were confirmed using t-test or Mann-Whitney U test. Correlations were analyzed using Spearman´s rank correlation.

Results

Characteristics of the study population

A total of 63 individuals living in a village endemic for *Loa loa* were eligible and thus screened. Specific IgG4 to filarial antigens, ocular passage and midday thick blood smear were assessed in all subjects. Participants were divided into 3 categories: infected Microfilaremic (MF+, n=19), Infected Amicrofilaremic (MF–, n=28) and uninfected Endemic controls (EN, n=17). Randomly selected MF+ (n=10) subjects and age-, gender- and household-matched EN (n=10) individuals were asked to donate blood for cellular immunological analysis. As there was material in the field for a total of 32 study subjects, ten matched MF– plus two more randomly selected individuals were included in the MF– group (n=12).

As shown in table 1, groups had similar demographic data. Regarding hematological values, no significant differences were found for hemoglobin levels or white blood cell counts. However eosinophil numbers were markedly higher in MF+ and MF– individuals compared to EN. The MF+ and MF– were more frequently co-infected with other helminths, such as *Mansonella perstans* or intestinal helminths. However, this difference did not reach statistical significance.

Cytokine responses to PHA and BmA differ between the different clinical groups

As shown in figure 1A, compared to EN, MF– had significantly higher levels of mitogen-induced Th2 cytokines, IL-5 (median [IQR] for MF– 692 [401-897] pg/mL and EN 228 [118-417] pg/mL; p=0.0018) and IL-13 (for MF– 1100 [428-1472] pg/mL and EN 384 [277-640] pg/mL; p=0.017). Similarly, in response to filarial antigen, BmA (figure 1B), IL-5 levels were higher in MF– (295 [193-533] pg/mL) versus EN (75 [31-200] pg/mL; p=0.007) and also higher compared to MF+ (104 [62-172] pg/mL, p=0.003). BmA-specific IL-13 was also significantly higher in MF– than in MF+ (291 [163-617] pg/mL and 102 [44-179], respectively, p=0.004). These data indicate a shift towards Th2 in MF– individuals in response to polyclonal stimulation and in particular to filarial antigen. Moreover, IFN-γ production was significantly lower following BmA stimulation in both MF– and MF+ compared to EN (for MF– 2.5 [2.5-2.5] pg/mL, MF+ 2.5 [2.5-8.2] pg/mL and EN 20 [18-26] pg/mL; both MF– vs. EN and MF+ vs. EN p<0.001), suggesting that the presence of microfilariae is associated with lower Th1 and Th2 cytokine production (figure 1B). IL-10 production in response to PHA was not different between the three groups, whereas the response to BmA showed significantly higher levels of this cytokine in MF– compared to both EN and MF+ (for MF– 96[87-248] pg/mL, EN 55[10-121] pg/mL and MF+ 62[45-101] pg/mL; p=0.007 and p=0.025 respectively). IL-17 production was markedly lower in both infected

MF+ and MF– groups compared to EN in response to PHA (for MF+ 170 [91-404] pg/mL, MF– 378 [129-676] pg/mL and EN 3039 [841-6437] pg/mL; p <0.001 and p=0.001, respectively) and BmA (for MF+ 10 [5-10] pg/mL, MF– 10 [5-15] pg/mL and EN 55 [10-121] pg/mL; both p<0.001). Cytokine levels in supernatants of medium-stimulated cells were below the detection limit of the assay in almost all samples.

Table 1. Characteristics of study population.

	Infected Microfilaremic (MF+)	Infected Amicrofilaremic (MF–)	Endemic Controls (EN)	p-value*
N	10	12	10	
***Loa loa* infection status**				
Blood microfilaria *(n)*	10	0	0	
Positive IgG4 *(n)*	8	8	0	
Ocular passage *(n)*	10	10	0	
Microfilaria per mL *(mean (range))*	4531 (50–9700)	-	-	
Demographic data				
Age in years *(mean ±SD)*	34.2 ±11.3	32.2 ±12.5	31.9 ±8.5	ns
Weight (kg) *(mean ±SD)*	64.0 ±12.5	69.8 ±16.8	70.6 ±13.7	ns
Height (cm) *(mean ±SD)*	164 ±5.8	165 ±7.6	164 ±15.3	ns
Gender *(M/F)*	5/5	6/6	5/5	
Hematological data				
Hemoglobin (*g/dL) (mean ±SD)*	13.6 ±1.74	14.1 ±5.20	14.5 ±3.16	ns
Eosinophilia (*%) (mean ±SD)*	21.6 ±5.9	19.9 ±9.4	5.9 ±3.6	p<0.001
Other helminth infections *n (%)*	6 (60%)	6 (50%)	3 (30%)	ns
Mansonella perstans	4 (40%)	4 (30%)	1 (10%)	ns
Intestinal helminths *n (%)*	5 (50%)	4 (30%)	20 (20%)	ns
Trichuris trichiura (n)	4	3	1	
Ascaris lumbricoides (n)	4	4	2	
Ancylostoma duodenale (n)	3	3	1	
Schistosoma haematobium n (%)	0	1(10%)	0	ns

**p-value calculated by Oneway ANOVA; ns = not significant*

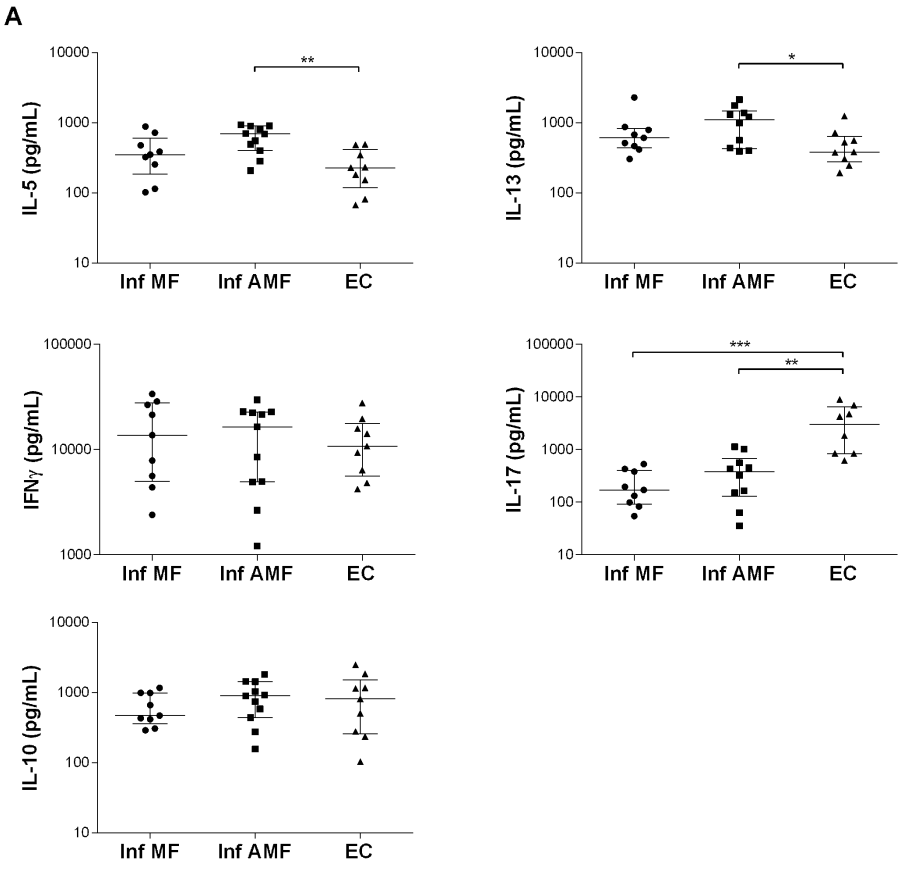
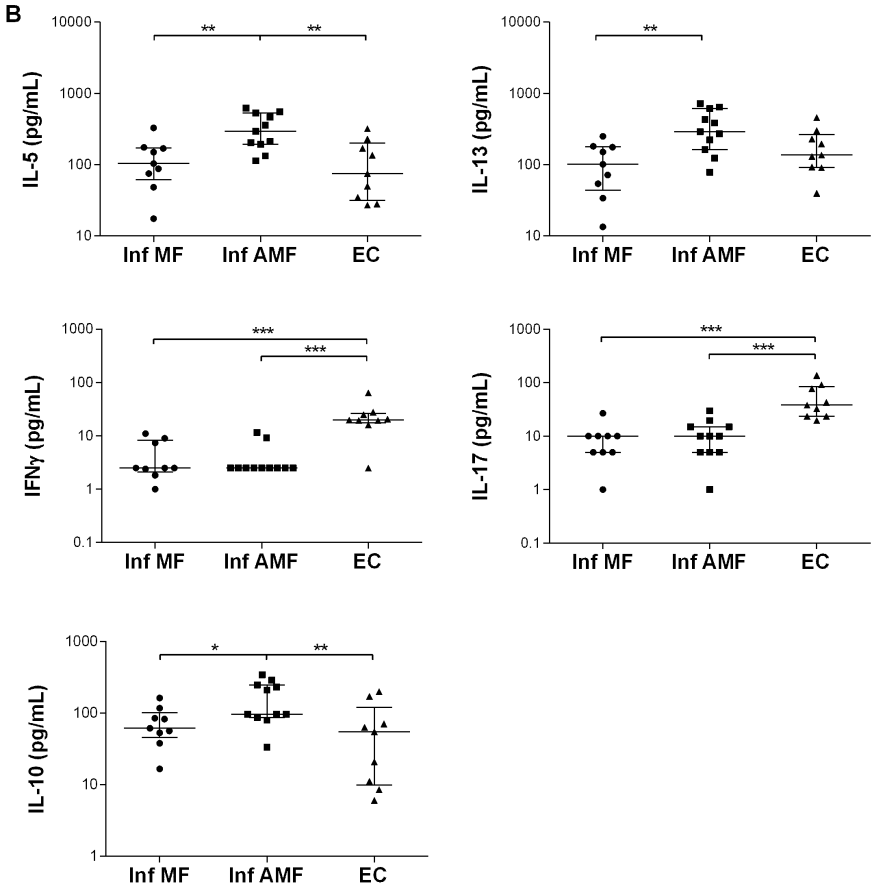


Figure 1. Altered filarial antigen specific and mitogen induced cytokine responses in microfilaremic and infected amicrofilaremic individuals. PBMC were stimulated for 72 hours with PHA (A; above) and BmA (B; right page) to assess cytokine production in culture supernatants. Cytokine levels were compared between microfilaremic individuals (MF+; circles), infected subjects who were amicrofilaremic (MF–; squares) and endemic controls (EN; triangles). For each group, the horizontal lines show median [IQR] and statistical differences are indicated as *p<0.05, **p<0.01and ***p<0.001



Circulating CD4⁺CD25^{hi}FOXP3⁺ T cell frequencies are higher in *Loa loa* infected subjects

To compare the frequencies of circulating Tregs, *ex vivo* CD25^{hi}FOXP3⁺ T cells were measured by flow cytometry in MF+, MF– and EN patients. As shown in figure 2, frequencies of Treg cells (as % of CD4⁺) were higher in MF+ and MF– compared to EN (mean±SD for MF+ 1,54 ± 0,64, MF– 1,06±0,43 and EN 0,54±0,45; p=0.012 and p=0.016 respectively). Moreover, levels of Treg cells were higher in MF+ than in MF– (p=0.047). These data show an association between *Loa loa* infection and expansion of Treg and suggest that within infected individuals, the presence of microfilaria in the bloodstream might lead to greater expansion of Treg cells.

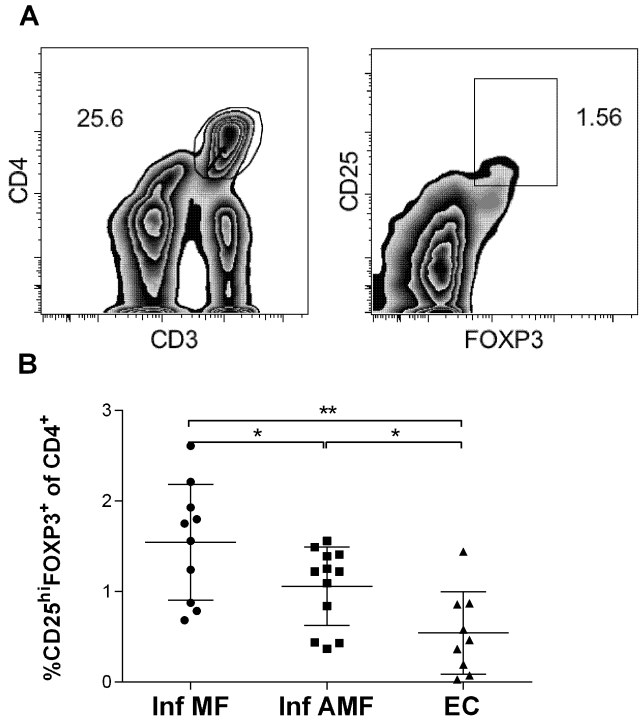


Figure 2. Higher Treg frequencies in loiasis. PBMC were fixed and stained for flow cytometry to analyse CD25 and FOXP3 expression. (A) A representative example is shown for the gating strategy of Tregs characterized as the $CD25^{high}FOXP3^{+}$ subset of $CD4^{+}$ T cells within PBMC (B) Percentage of Treg in MF+ (circles), MF– (squares) and EN (triangles) groups are shown with horizontal lines representing means. Statistical differences are indicated as *p<0.05 **p<0.01.

Intracellular IL-4, IFN-γ, IL-10 and IL-17 production by $CD4^{+}$ T cells and correlation with circulating Tregs

The frequency of $CD4^{+}$ T cells producing IL-4, IFN-γ, IL-10 and IL-17 (as % of total CD4) after stimulation with PMA plus ionomycin was measured by flow cytometry. As shown in figure 3A, the percentage of IFN-γ producing cells was significantly lower in MF+ compared to EN (mean±SD for MF+ 7.07±5,53 and EN 13.73±6.57, p=0.028) and although it also appeared to be lower in MF– (8.83±4.83) than EN, this difference did not reach statistical significance (p=0.063). The percentage of IL-4 producing cells was not different between the three groups (for MF+ 9.04±6.74, MF– 9.35±5,29 and EN 8,63±4.70; figure 3B), nor that of IL-10 producing cells (for MF+ 5.49±4.82, MF– 6.69±4.13 and EN 7.20±4.15; figure 3D). However, when the ratio of Th2/Th1 was analyzed, the MF+ group had the strongest Th2 skewing followed by MF–, with the lowest ratios of IL-4 / IFN-γ in EN, as shown in figure 3C (median [IQR] for MF+ 1.4[0.7-2.2], MF– 1[0.8-1.6] and EN 0.6[0.5-0.8]; p-values: 0.017 and 0.009 respectively). Furthermore, frequencies of Th17 cells were significantly lower in both MF– (p=0.008) and MF+ (p=0.033) compared to EN (mean±SD for MF+ 1.25±1.17, MF– 1.15±0.60 and EN 2.92±1.97; figure 3E).

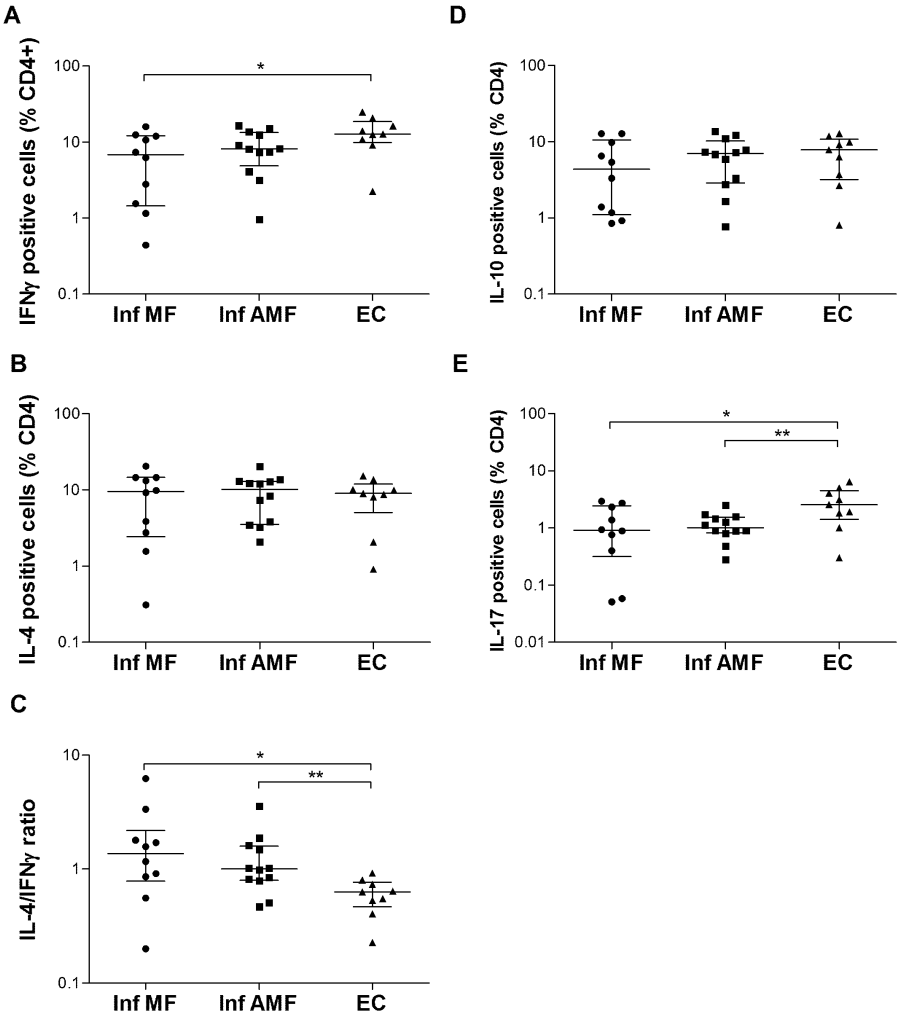


Figure 3. Intracellular cytokine production after PMA stimulation. PBMC were stimulated for 6 hours with PMA-ionomycin, and for 4 hours more in the presence of Brefeldin A, after which intracellular cytokines were detected by flow cytometry. MF+ (circles), MF– (squares) and EN (triangles) were compared for the percentages of A) IFN-γ^+, (B) IL-4^+, (D) IL-10^+, and (E) IL-17^+ producing $CD4^+$ T cells and in (C) the ratio of IL-4^+ to IFNγ^+ cells is shown. Statistical differences are indicated as *p<0.05 **p<0.01 ***p<0.001

No correlations were found between Tregs and either Th1 or Th2 responses. Interestingly, there was a statistically significant positive correlation between proportion of Treg cells and the frequency of IL-17 producing CD4+ T cells in the EN group (Fig 4A; r = 0.83, p=0.008) but no such correlation was found in the infected groups in either pooled (MF– + MF+) or separate (MF– or MF+) analysis (pooled analysis r = -0.16, p=0.47, figure 4B).

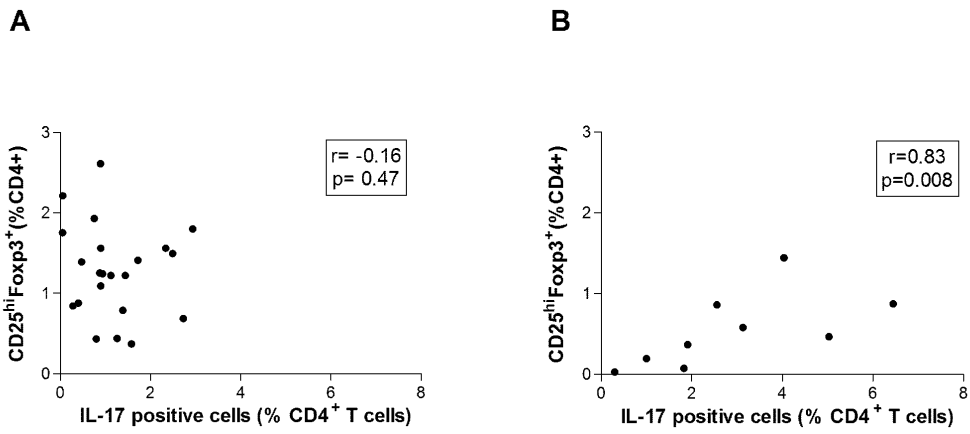


Figure 4. Correlation between Tregs and IL-17 producing CD4+ T cells. The correlation is shown between Treg frequencies (x-axis) and production of IL-17 by CD4+ T cells in response to PMA (y-axis). Spearman's Rank test was used to measure correlation, of which the coefficient r and p-values are depicted.

Discussion

T cell hyporesponsiveness seems to play a major role in the ability of parasites to evade host immunity and consequently maintain chronic infection[25]. Several studies have shown that Treg cells are key players in the downregulation of effector cells[26]. Helminth infections such as *Heligmosomoides polygyrus*[27] and *Schistosoma mansoni*[28] have been shown to lead to increased number of Tregs in murine models. Furthermore, infection with *Brugia malayi* larvae leads to the expansion of Foxp3$^+$ Tregs[15] and removal of Tregs has been shown to reverse hyporesponsiveness induced by *Litomosoides sigmodontis*, and to restore parasite killing[29]. These data support the hypothesis that filarial infections can lead to Treg expansion, which in turn suppresses host immune responses allowing the long-term survival of these parasites within their immune-competent hosts. In this study, we showed that infections with the tissue dwelling filarial parasite *Loa loa* are associated with increased frequencies of CD25^{hi}FOXP3$^+$ T cells in humans. Moreover, a significantly higher frequency of Treg cells was observed in MF+ compared to MF–, suggesting that microfilaria in the bloodstream may be stronger inducers of Treg expansion. The increase of CD4+CD25^{hi}FOXP3$^+$ T cells in lymphatic filariasis has been shown by flow cytometry[30] and by mRNA expression[16] by comparing microfilaremic subjects with uninfected controls. In another chronic helminth infection, schistosomiasis, the frequency of Tregs, characterized by high expression of CD25, was reported to be elevated and treatment was shown to lead to a significant reduction in the number of Tregs indicating that the presence of *Schistosoma mansoni* was driving regulatory T cell expansion[31]. However, a recent cross-sectional study of regulatory T cells in *Schistosoma haematobium* infections using the signature marker FOXP3, showed that only in infected children but not in infected adults the intensity of infection was correlated with number of Tregs[32].

Impaired Th1 responses are thought to be characteristic of filarial infections, though some studies have shown that Th2 responses can also be suppressed[19,33,34]. In *Loa loa* infection with circulating microfilaria, T cell unresponsiveness including Th1 and Th2, has already been reported[12]. When analyzing culture supernatants, our data are consistent with previous studies; specific antigen stimulation showed a shift towards Th2 with impaired Th1 in MF–. In addition, in MF+ subjects both Th1 and Th2 were low. Therefore, in *Loa loa* infection, microfilaremia seems to be associated with more profound immunoregulation. This situation would allow the survival of microfilariae, the life cycle stage that is crucial for maintaining parasite transmission.

Polyclonal stimulation with PHA also indicated stronger Th2 skewing in infected subjects. Globally, this was supported by data from intracellular cytokine analysis of CD4$^+$ T cells following stimulation with PMA plus ionomycin which showed a

strong skewing of responses towards Th2 and away from Th1 in MF+ and MF– subjects compared to EN. While there is consent regarding Th1 impairment in helminth infection and its direct association with regulatory T cell network, modulation of Th2 responses seems to be more complex. In MF–, Tregs may contribute to T cell hyporesponsiveness, but in MF+ subjects a more profound anergic state might exist. A recent study in murine chronic schistosomiasis showed that the characteristic Th2 cell hyporesponsiveness was linked to an increased E3 ubiquitin ligase GRAIL[35]. As a result of continued antigen stimulation, the GRAIL expression led to lymphocyte anergy, a mechanism referred to as adaptive tolerance, where persistent antigen stimulation is needed in order to maintain the non-responsiveness. Interestingly, in one study on lymphatic filariasis the expression of the cbl-b and c-cbl proteins which belong to the RING family of the E ubiquitin ligase family was observed to be upregulated in PBMCs of microfilaremics and might be responsible for T cell anergy[16].

In this study IL-17 production was significantly suppressed in infected individuals, both MF– and MF+ subjects. It is known that Th17 and Treg are linked and that they arise in a mutually exclusive fashion[36], which is supported by our data showing that the frequencies of IL-17 producing $CD4^+$ T cells are inhibited during infection while regulatory T cells are upregulated. The role of Th17 subset in helminth infection remains poorly understood. It has been shown that the induction of immunopathology in murine and human schistosomiasis was correlated with increased levels of IL-17 and number of Th17 cells[37,38] and in one study in human lymphatic filariasis, the elevated Th17 responses in lymphedema patients suggest that Th17 cells may have the potential to play a role in mediating pathology during filarial infections[18]. The fact that *Loa loa* infection can suppress antigen-specific and polyclonal Th17 responses by activating Tregs may prevent the development of severe pathology. In line with this, in an autoimmune model, TGF-β mediated Th17 suppression has been observed in mice infected with *Fasciola hepatica,* which results in attenuated experimental encephalomyelitis[39]. Interestingly, there was a positive correlation between Treg and Th17 cells in the uninfected endemic control subjects which was not seen in infected individuals raising the possibility that only during chronic helminth antigen challenge the reciprocal control of Treg and Th17 starts to take shape.

In a recent study, the frequency of cytokine producing cells in whole blood, without any activation, was studied in lymphatic filariasis in Mali[30] where microfilaremics were compared with uninfected subjects. In contrast to our findings, the number of IL-17 producing $CD4^+$ T cells was higher in microfilaremic subjects. In the study by Metenou and colleagues[30], the cells were not stimulated whereas in our study we have used the standard method of PMA stimulation in order to detect cytokine-producing cells. This would suggest that immune profiles of *ex vivo* unstimulated cells are different from what is seen in the same cells when

activated to produce cytokines. Indeed, the same group has recently shown that when stimulated with antigen, IL-17 producing cells were found to be lower in microfilaremic subjects[40]. However, it is also important to consider that high levels of Th17 in EN in Gabon might be due to high fungal exposure as already reported for our area[41], where humidity often exceeds 70%, as compared to the dry environment in Mali. It is well known that Th17 responses develop in response to fungal extracts[42] and the activation of Treg cells during filarial infection could lead to a downregulation of these Th17 cells. A question that remains unanswered is whether the elevated Th17 in EN, might suggest a role for Th17 cells in anti-filarial immunity; it should be noted that there is so far no evidence for a role of Th17 cells in anti-filarial immune responses.

Taken together, our results show that in an area endemic for *Loa loa* infection, the classic Th2 skewing is seen when considering the Th1/Th2 balance but with respect to the regulatory T cells and Th17 cell axis, *Loa loa* infection is associated with increased Treg but decreased Th17. Interestingly, it is not known what the clinical consequences of such lowered Th17 cells are for inflammatory disorders, in general, or for immunity to infections, in particular.

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