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Immunomodulation in chronic Loiasis−Eosinophils, Basophils and Myeloid-Derived Suppressor Cells in *Loa loa* Infection and its Treatment in an Endemic Setting

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Abbreviations

AE	adverse event
APC	allophycocyanin
CBA	cytometric bead array
CERMEL	Centre de Recherches Médicales de Lambaréné
CFSE	carboxyfluorescein succinimidyl ester
CX3CR1	CX3C motif chemokine receptor 1
Cy5.5	cyanine5.5
Cy7	cyanine7
DALYs	disability-adjusted life years
DEC	diethylcarbamazine
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCS	flow cytometry standard
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
G	gravitational units
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
HIV	human immunodeficiency virus
HLA-DR	human leukocyte antigen-DR isotype
IgE	immunoglobulin E
IgG	immunoglobulin G
IFN-γ	interferon gamma
IL	interleukin
IQR	interquartile range
LAMP	loop-mediated isothermal amplification

LL-	*Loa loa*-uninfected
MACS	magnet-activated cell sorting
MDA	mass drug administration
MDSC	myeloid-derived suppressor cells
mf/ml	microfilariae per milliliter
MF	microfilariae
MF-	amicrofilaraemic
MF+	microfilaraemic
MFI	median fluorescence intensity
min	minutes
M-MDSC	monocytic myeloid-derived suppressor cells
NTD	neglected tropical disease
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-L1	programmed death-ligand 1
PE	phycoerythrin
Pen-Strep-Glu	penicillin-streptomycin-glutamine
PerCP	peridinin chlorophyll protein
PMN-MDSC	polymorphonuclear myeloid-derived suppressor cells
RANTES	regulated on activation, normal T cell expressed and secreted
RAPLOA	rapid assessment procedure for *Loa loa*
RDT	rapid diagnostic test
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute medium
SAE	severe adverse event
SI	separation index
spp.	species pluralis
SSC	side scatter
TBS	thick blood smear

TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
Treg	T regulatory cell
TSLP	thymic stromal lymphopoietin
WHO	World Health Organization

1 Introduction

In chronic loiasis—a neglected filarial disease affecting over 10 million people in Central and West Africa—adult *Loa loa* worms can migrate through the body for decades and release larvae (microfilariae) into the blood stream that can reach densities of over 100,000 microfilariae per milliliter (mf/ml), suggesting exemplary immunotolerance.[1,2] In contrast, the treatment of individuals with high levels of microfilariae can lead to a severe, and in some instances fatal, inflammatory response and immune-mediated encephalitis due to the rapid death of microfilariae.[3,4] Recent epidemiologic research initiated a paradigm shift towards the recognition of loiasis as a major public health problem that causes significant morbidity and mortality in affected populations.[5–7] However, research addressing the underlying immunology of this disease remains scarce. This thesis aims to shed light on the dichotomy of immunomodulation in loiasis by investigating the role of innate immune cell subsets—eosinophils, basophils and myeloid-derived suppressor cells—and associated circulating cytokines in the chronic infection and treatment of *Loa loa* in an endemic region in Gabon. The current state of research concerning *Loa loa* and its immunology as well as the nature and role of eosinophils, basophils and myeloid-derived suppressor cells is reviewed hereinafter. A summary of the overall research question, and its sub-questions, addressed in this thesis, is provided at the end of this chapter (page 18).

1.1 Loiasis—a truly neglected tropical disease

1.1.1 Epidemiology and public health significance

Around 30 million people live in high- and intermediate-risk areas for loiasis in the Central and West African equatorial rain forest region and it is estimated that more than 10 million are infected.[1,2] Gabon and Equatorial Guinea are the most endemic countries, with nearly their whole area falling into the high-risk category (prevalence of eye worm history > 40%) while the majority of people at risk live in the Democratic Republic of the Congo (DRC) and Cameroon (Figure 1.1).[2]

Loiasis should rightfully be classified among the neglected tropical diseases (NTDs), a group of chronic and debilitating conditions disproportionally affecting the world's poorest and most vulnerable populations, although it has not yet been considered officially.[8] NTDs, amongst others lymphatic filariasis, onchocerciasis, leishmaniasis and soil-transmitted helminths, globally cause a loss of 56.6 million disability-adjusted life years (DALYs)—a disease burden higher than that of malaria or tuberculosis, which is likely to increase in the future.[9,10] These estimates do not yet include the detrimental

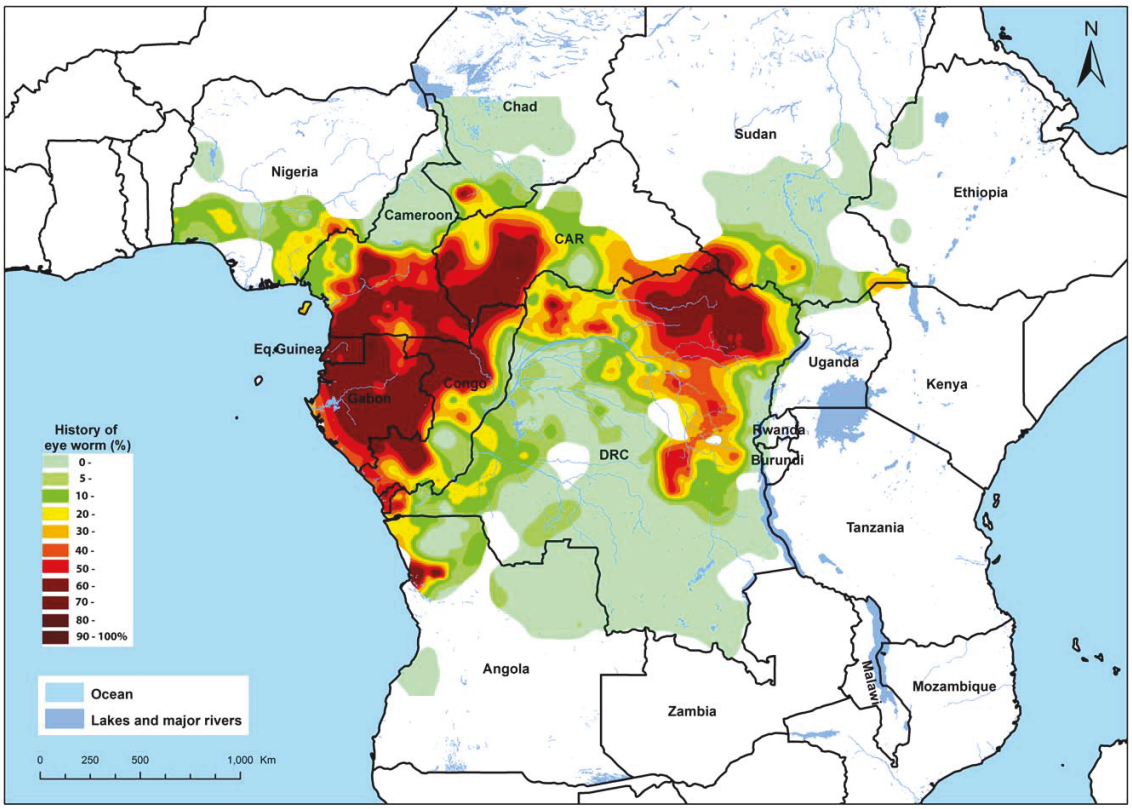

Figure 1.1: Map of the estimated prevalence of eye worm history in Africa
Adapted from Zouré, Wanji et al., 2011.[2]

economic impact of NTDs that result from reduced child development, education, agriculture and economic productivity, trapping affected populations in a cycle of poverty and disease.[10,11] In endemic countries, reducing the burden of NTDs forms a major focus of the World Health Organization (WHO) sustainable development goals for 2030, an initiative of all United Nations (UN) member states to globally ensure healthy life and promote well-being for all.[12–14] NTDs have partly been recognized as a public health issue by the WHO and there are now some very effective control programs in place—e.g. mass chemopreventive therapy for soil-transmitted helminth infections, schistosomiasis, onchocerciasis and lymphatic filariasis.[15–18] However,—given that control of NTDs has proven a highly cost-efficient way to alleviate significant disease burden and poverty—more advocacy, funding, research, treatment access and integrated control programs are urgently needed.[13,19]

The perception of loiasis as a benign condition has been challenged by researchers for more than 70 years.[20] Early publications described that in some areas, loiasis can be the second most common reason for medical consultation and a serious public health concern.[21–23] However, loiasis only gained some momentum on the international research agenda when severe adverse events (SAEs) occured in highly *Loa loa*-microfilaraemic

individuals treated with ivermectin, impeding mass drug administration programs against onchocerciasis and lymphatic filariasis using this drug in areas co-endemic for loiasis.[24] Both diseases are targeted for elimination by the WHO and currently 1.2 million *Loa loa*-infected individuals are estimated to be at high risk of experiencing life-threatening SAEs if treated with ivermectin.[25,26]

Recent publications provide evidence for the importance of loiasis as a disease and public health issue in its own right: In a systematic review of case reports including 329 infected individuals, 47% presented with atypical clinical manifestations, encompassing a wide range of symptoms that have traditionally not been associated with loiasis.[27] Residents of loiasis-endemic areas were most at risk of severe manifestations. A retrospective cohort study, conducted over a 15-year period in Cameroon found that participants with high *Loa loa*-microfilaraemia died significantly earlier than those without microfilaraemia.[6] The population-attributable fraction of mortality associated with presence of microfilaraemia was 14.5%. A recent investigation from the Republic of Congo yielded similar results.[7] Finally, a recent cross-sectional study conservatively estimated the morbidity-based burden of disease associated with loiasis at over 400 DALYs per 100,000 inhabitants in a rural population in Gabon and approximately 80 DALYs per 100,000 inhabitants at the country-level.[5] In comparison, soil-transmitted helminths are estimated to cause 15 DALYs per 100,000 inhabitants in Gabon; yet they are widely recognized as a public health problem and a national control program is in place.[28,29] The study also revealed that the majority of DALYs accumulate in the most economically active age groups (15–49 years). Particularly, eye worm migration caused significant impairment of household activities and work. Nevertheless, it seems likely that the study still underestimated the true burden of disease, since mortality data were not available, and no atypical symptoms were included in the calculation of DALYs.

In conclusion, loiasis expands well beyond a benign condition, causing significant morbidity and mortality in rural populations in Central and West Africa. Large multicenter longitudinal studies would be necessary to fully assess the effect of loiasis in these endemic populations.[1] Drugs and vector control are available tools for public health interventions.[30–33] However, to the author's knowledge, no specific control efforts have been initiated until today. Given the evidence above, loiasis represents a *truly* neglected tropical disease and is long overdue for recognition as a significant public health concern in its own right.[5,28] Researchers urgently call for inclusion of loiasis in the WHO's formal list of NTDs to generate funding for targeted research and integrated control programs.[1,5,28,34–36]

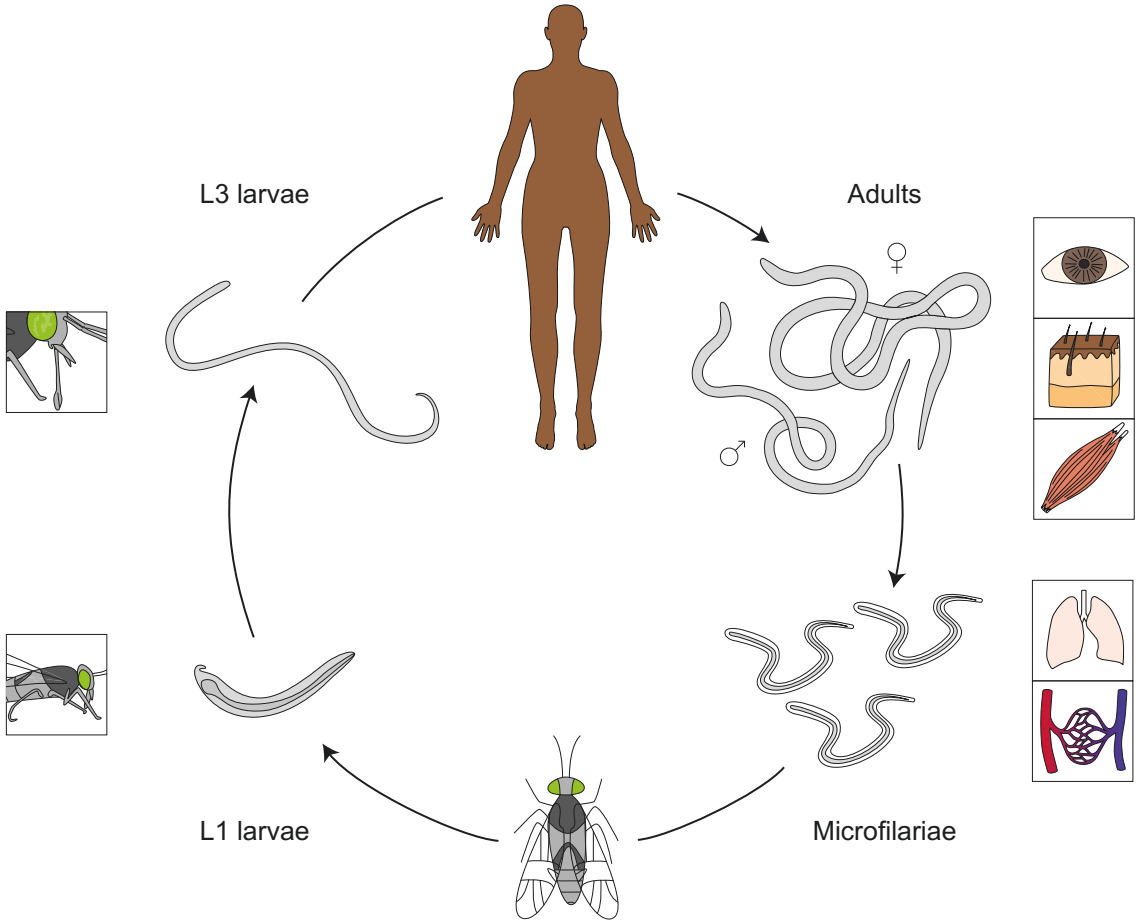

Figure 1.2: Life cycle of *Loa loa*
As many as 200 third-stage infective larvae (L3) can migrate to the proboscis and enter the dermis during the blood-meal of an infected *Chrysops spp.*[37] L3 develop into young adults (macrofilariae) within 19 to 50 days and keep growing up to 300 days after infection.[38,39] Mature adults can measure over 6 cm and migrate through subcutaneous connective tissues and between the fascial layers of somatic muscles for decades.[40–43] They cause the pathognomonic eye worm (Figure 1.3) and can enter peritoneal and pleural cavities.[44–46] After mating, female adults produce up to 22,000 microfilariae (MF) per day.[47] Prepatency—the time between inoculation of L3 larvae and detection of MF—is around 150 days.[39,40,48] MF migrate through the lymphatic system and accumulate in the pre-capillary arterioles of the lungs from where they periodically enter the peripheral blood stream, probably aligned to the diurnal rhythm of the host's body temperature.[40,49–51] Here, microfilaraemia peaks around 11:00–16:00—when *Chrysops spp.* are most active—and can reach over 100,000 mf/ml.[50,52–54] Upon the bite of a *Chrysops spp.*, MF enter the fly's stomach and begin to penetrate its wall. They migrate into the fly's abdominal fat bodies, where they develop into L1 larvae.[55] They reach L3-stage within 7–12 days after infection and proceed to the fly's head to infect another human.[46,56,57] Figure by Gerrit Burger.

1.1.2 Parasitology

Loa loa is a nematode (roundworm) helminth belonging to the filarioidea superfamily. Other notable members are *Brugia malayi*, *Brugia timori* and *Wucheria bancrofti* causing lymphatic filariasis (elephantiasis) and *Onchocerca volvulus* causing onchocerciasis (river blindness).

Human infection with *Loa loa* occurs through diurnal bites by *Chrysops spp.* tabanid flies.[58,59] The two main vectors *C. silacea* and *C. dimidiata* have been found in the greater part of the African tropical equatorial rain forest and their geographical distribution overlaps significantly with *Loa loa* prevalence.[33,60] The flies dwell in light-shaded forest canopies, prefer intermediate temperature, humidity and tree canopy coverage and breed in densely shaded, slow-flowing streams in fine sand and mud.[60–64] The strong link between their defined ecological niche and *Loa loa* prevalence is best illustrated in the north-eastern Democratic Republic of Congo, where the region's special ecology (Congo river basin with dense, edaphic forests; hot and humid conditions) is associated with both low *Chrysops spp.* and *Loa loa* prevalence (Figure 1.1).[65] *C. silacea* and *C. dimidiata* predominantly feed on human blood and biting peaks between 9:00 and 16:00 correspond well to the diurnal periodicity of peripheral blood *Loa loa*-microfilaraemia.[66,67]

Figure 1.2 illustrates the detailed life cycle of *Loa loa*. Briefly, third-stage infective larvae (L3) enter the dermis during the bite of a *Chrysops spp.*, where they begin to develop into adult worms (macrofilariae). Adult worms migrate through subcutaneous and perifascial connective tissues for decades and cause the pathognomonic eye worm symptom. Female adults produce microfilariae (MF) that accumulate in the pre-capillary arterioles of the lung. From here they periodically enter the peripheral blood stream. Upon the blood-meal of another *Chrysops spp.*, MF enter the fly and develop into L3 larvae, after which they are ready to infect another human.

1.1.3 Disease

Forest exposure is the main risk factor for loiasis, since the majority of infections is acquired in the habitat of the vector.[68–73] Thus, rural populations and members of lower socioeconomic groups (e.g. farmers and hunters) are at increased risk.[72–75] Men are more frequently infected than women, which could be related to behavioral (higher forest exposure in men) or hormonal factors.[72,73,76–79] Several studies have shown a correlation between age and *Loa loa* infection, presumably indicating its chronic and cumulative nature.[5,72,80–83] While some *Loa loa*-infected individuals can harbor high levels of microfilariae circulating in the bloodstream, around 70% remain amicrofilaraemic (occult infection).[34,80,84] *Loa loa* prevalence and microfilarial load are correlated[72,85–87] and stable over time at both the individual and community levels.[88–90] The incubation period is

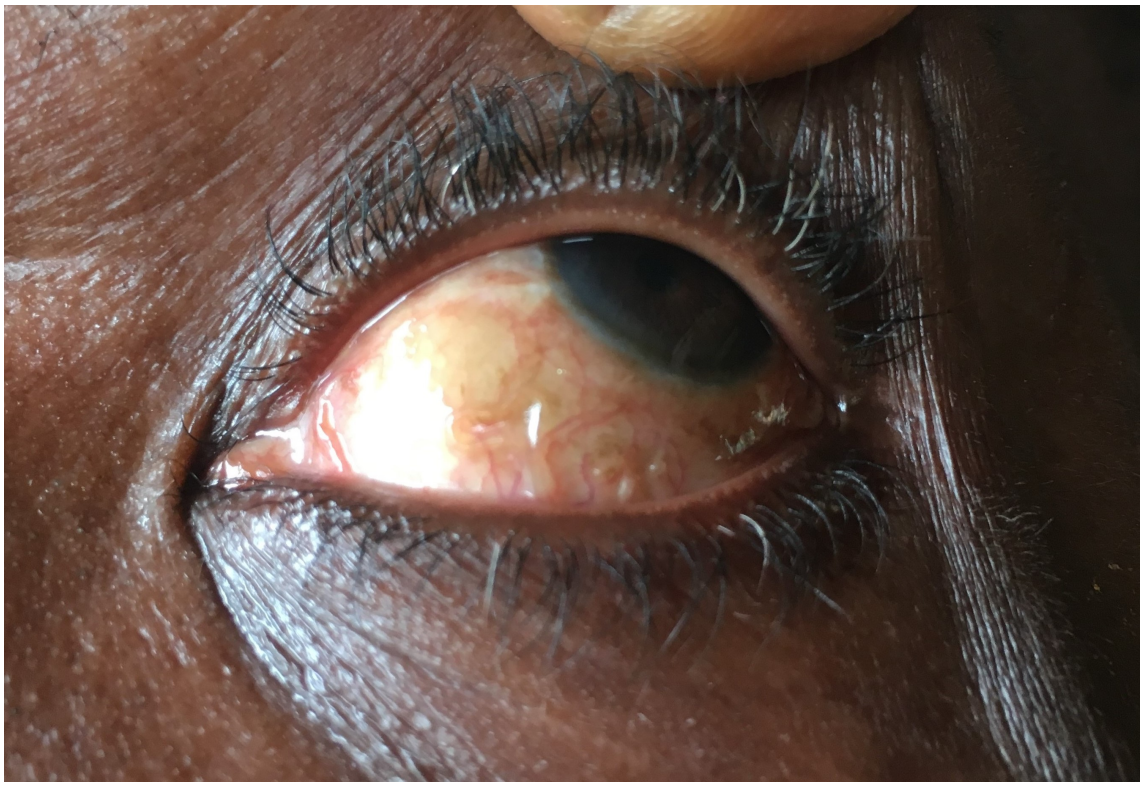

Figure 1.3: Eye worm—subconjunctival passage of an adult *Loa loa* filaria
A 60-year-old male study participant reported an ongoing episode of eye worm with ocular pain and irritation. Upon examination an adult *Loa loa* filaria during subconjunctival passage could be identified in the inferior-nasal quadrant of the left eye, accompanied by conjunctival injection and chemosis. Informed written consent for publication was obtained from the study participant and approved by the Institutional Ethics Committee (reference CEI-022/2018) at the Centre de Recherches Médicales de Lambaréné (CERMEL). Photograph by Gerrit Burger.

typically around 10 months, but can vary from five months to as long as 20 years.[43,91]

Eye worm—subconjunctival passage of an adult *Loa loa* filaria (Figure 1.3)—is the most striking and pathognomonic symptom of loiasis.[92] It causes pain, local inflammation, periorbital swelling and temporal vision impairment and often resolves within one to four days.[5,80] Complications include filarial migration into the anterior or posterior chamber of the eye, causing vitreous hemorrhage, posterior chamber opacification, uveitis, hemorrhagic retinopathy and/or retinal detachment and thus long-term vision impairment or loss.[27] Traditional treatments in rural populations include the application of garlic, onion, lemon juice, leaf extracts or chili to the eye as well as removal (attempts) with razor blades, palm tree thorns, old syringes or bamboo sticks—sometimes leaving permanent visual damage.[34,93] A second characteristic manifestation are transient migratory angioedemas named Calabar swellings.[94] The non-pitting, itchy edemas appear most commonly on the wrists, forearms and legs.[95,96] They appear and disappear spontaneously in different intervals and locations and are presumably caused by the host's inflammatory

response against antigens or microfilariae released by adult worms.[59,97] Other classical presentations include pruritus, urticarial rush, and arthralgia.[80,91,95,96,98,99]

A wide range of other symptoms have been associated with *Loa loa* infection as Buell, Whittaker et al.[27] summarized in a systematic review of case reports including 329 infected individuals: Nervous system-related manifestations include spontaneous encephalitis (with MF found in the cerebrospinal fluid in some cases), severe headaches and peripheral nerve palsies. *Loa loa*-induced membranous glomerulonephropathy can cause symptoms ranging from asymptomatic proteinuria to severe nephrotic syndrome and end-stage renal failure.[27] MF have been found in renal biopsies and urine. Cardiac manifestations include signs of heart failure and endomyocardial fibrosis.[27] Respiratory system manifestations include pleural effusion or pulmonary fibrosis and MF can be detected in both pleural fluid and broncho-alveolar lavage in some cases. Gastrointestinal manifestations reported consist of splenic lesions with splenomegaly, ascites, hepatitis, bowel obstruction and intestinal polyps. Reproductive system manifestations include painful migration of adult worms through the testicles or female breasts. MF have been found in follicular fluid in infertile women, in routine cervical smears and the placenta.[100] Beyond common arthralgia, acute septic joint has been described as a complication with MF sometimes found in articular fluid.[27] Recent cross-sectional studies from the Republic of Congo have revealed associations between *Loa loa* microfilaraemia and chronic conditions, such as proteinuria, arterial stiffness and cognitive impairment, i.e. reduced MoCA (Montreal Cognitive Assessment) scores.[101–103] However, the real extent of these non-classical and chronic manifestations of loiasis—and potentially other manifestations not yet associated with *Loa loa* infection—remains to be further studied.

1.1.4 Diagnosis

Clinical diagnosis of loiasis can be established by observation or history of eye worm. Suspicion should be raised in (temporary) residents of endemic areas presenting with typical clinical findings such as Calabar swellings, pruritus, arthralgia, or marked eosinophilia without any other plausible cause.

Microfilaraemic infection

Microfilaraemic patients can be diagnosed by microscopic examination of peripheral blood drawn between 10:00 and 15:00 (see Section 2.3.2). While the most common method is examination of Giemsa-stained thick blood smears (TBS), other methods such as fluorescence techniques have been proposed.[104,105] To increase sensitivity for low-microfilaraemic samples, concentration techniques involving red blood cell lysis or filtration can be employed.[106–109] The average level of MF may be higher in capillary than in

venous blood samples.[110] The LoaScope—a smartphone-based point-of-care diagnostic tool that uses video microscopy and motion detection to quantify microfilariae from capillary blood—has proven to be a reliable and rapid new technique to identify highly microfilaraemic individuals,[111–113] while a similar platform has recently been established.[114] Other new approaches for direct MF quantification include modified automated handheld counters or flow cytometry.[115,116] Finally, MF can be detected and in some cases quantified by molecular methods such as (quantitative) polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP).[117–120] Circulating MF antigens as correlates of microfilarial density have been identified, which is a promising step towards quantitative point-of-care immunoassays.[121,122] Current serologic methods cannot be employed to reliably identify (particularly highly) microfilaraemic individuals.[123]

Amicrofilaraemic (occult) infection

Diagnosis of amicrofilaraemic (occult) infection can be challenging: Although serologic testing for immunoglobulin G (IgG) by enzyme-linked immunosorbent assay (ELISA) has been established early using crude worm extract, this technique has its limitations due to the availability of antigens and lack of standardization.[124,125] Testing for IgG against the recombinant *Loa loa* SXP-1 protein has been realized on different platforms such as ELISA, luciferase immunoprecipitation system (LIPS) and even as a lateral flow assay (LFA) that demonstrates high sensitivity and good specificity (see 2.3.3).[126–129] However, none of these techniques can differentiate between active and past infection, and cross-reactivity with other filarial species can be problematic in endemic settings.[125] PCR protocols have successfully been established to identify amicrofilaraemic infections.[130–132] LAMP has proven high sensitivity to detect *Loa loa* DNA from artificially spiked human blood.[133]

1.1.5 Therapy

Therapy for loiasis is challenging due to limited data on the efficacy of available drugs, the long duration of treatment in low-resource settings and adverse events occurring in highly microfilaraemic individuals. The choice of drug regimen depends heavily on microfilarial status and density, so peripheral blood microscopy must be performed before initiating treatment. The currently available drugs are diethylcarbamazine (DEC), ivermectin and albendazole.

Diethylcarbamazine (DEC)

DEC is the first-line therapy for loiasis, since it is the only agent with proven macrofilaricidal activity, with cure rates of approximately 50% after one or more cycles (21

days each, 8–10 mg/kg/d).[134] However, it also acts rapidly on microfilariae, limiting its use to individuals with low microfilaraemia who are less at risk of adverse events.[135] 2,000 mf/ml has been proposed as the safety threshold for DEC treatment, although data are limited.[136] Since adverse events can also occur in amicrofilaraemic (or lowly microfilaraemic) individuals, a graded dosing regimen is recommended initially. DEC should not be used during pregnancy or in co-infections with *O. volvulus*.[137] Access can be limited in travel clinics in Europe.[138] DEC can be administered preventively in temporary residents of endemic areas with long and intense exposure to the vector.[139]

Ivermectin

Ivermectin is strongly microfilaricidal (but probably not macrofilaricidal) according to several studies.[140–146] Indeed, microfilaraemia is reduced by 60% and 90%, one day and three weeks, respectively, after a single dose (150–200 μg/kg); and the levels remain decreased one year after treatment.[147] Whether this long-term effect is due to partly macrofilaricidal or sterilizing effects on adult worms remains unclear.[136] Although decrease in microfilaraemia is less rapid compared to DEC,[148] adverse events occur in highly microfilaraemic individuals.[3,140,141,149,150] Thus, safety thresholds between 8,000 mf/ml in outpatient settings up to 30,000 mf/ml in hospitalized patients under close monitoring have been proposed for treatment with ivermectin.[136] In the context of mass drug administration (MDA) programs against lymphatic filariasis and onchocerciasis, ivermectin reduces both the prevalence and intensity of *Loa loa* infections.[30–32,151,152]

Albendazole

Albendazole seems to lead to a milder decrease in microfilaraemia, beginning at 14 days after initiation of treatment and still apparent after one year.[153–156] It is thus considered to have an embryostatic, embryotoxic, or possibly macrofilaricidal (but no microfilaricidal) effect, although data are sparse and partly contradictory. Albendazole (200 mg twice daily for 21 days) can be administered in individuals to reduce high microfilaraemia prior to treatment with ivermectin.[136] The drug is generally well tolerated, and only few cases of possibly *Loa loa*-related encephalopathy after treatment with albendazole have been described.[157–159]

Other agents

A range of other drugs have been found to show some effect against *Loa loa*: Within the class of benzimidazoles (which includes albendazole), flubendazole inhibited microfilarial development in a mouse model, while clinical studies investigating mebendazole mostly did not find an effect on *Loa loa* microfilaraemia.[154,160–162] The c-Abl-like

tyrosine kinase inhibitor imatinib has shown some antimicrofilarial activity *in vitro*, and has been used to decrease microfilarial loads before definitive treatment with DEC in a recent case report.[163,164] The anti-helminthic levamisole was recently investigated in a randomized controlled trial and was found to transiently decrease microfilaraemia.[165] Interestingly, the anti-malarial drugs mefloquine and amodiaquine decrease *Loa loa* microfilarial activity *in vitro*, while human immunodeficiency virus (HIV)-infected adults receiving cotrimoxazole preventive therapy seem to be at a lower risk of *Loa loa* microfilaraemia.[163,166,167] As *Loa loa* does not harbor *Wolbachia* endobacteria, antibiotics such as doxycycline are ineffective in loiasis.[168]

Adverse events

Among the most common adverse events upon treatment of loiasis are pruritus, arthralgia and headache, which may occur in up to 45% of microfilaraemic individuals.[141,148] Severe adverse events are rare (estimated at 1–10% in individuals with microfilaraemia of 20,000–50,000 mf/ml after ivermectin treatment) but can be life-threatening and both frequency and intensity depend heavily on microfilarial density.[3,24,135,140,150] Other risk factors are male sex and possibly alcohol or cannabis consumption.[149,150] The most common SAEs are neurological (*Loa loa* encephalopathy) and include headache, stupor, coma, motor deficit and palpebral subconjunctival haemorrhages.[149,169] Non-neurological symptoms include myalgia, arthralgia, hematuria and microfilaruria.[149,170] Symptoms mostly develop between 24–48 h after initiation of treatment.[148,149,171–173] The case fatality rate of *Loa loa*-related SAEs after ivermectin treatment was estimated between 5.8% and 23.5% in the Democratic Republic of Congo and Cameroon, respectively.[149,171]

As not all individuals with high *Loa loa* microfilaraemia develop SAEs, different co-factors have been proposed: Mutations in the mdr-1 gene—encoding the drug-transporting P-glycoprotein, an essential part of the blood-brain barrier—can increase neurotoxicity of ivermectin in animals and might occur more often in humans who experienced *Loa loa*-related SAEs.[174–177] Co-infection with *P. falciparum*—although potentially damaging cerebral vascular endothelium—does not seem to increase the risk of SAEs.[178]

Prevention of adverse events

There are various strategies to prevent or mitigate adverse events upon treatment of loiasis: A graded dosing regimen is (empirically) used for DEC, but has not shown a significant reduction in adverse events for ivermectin.[179] Apheresis can lower microfilarial densities by around 30–67%, but is often not available.[170,180–185] Anti-histamines and corticosteroids—although repeatedly used—seem not to be efficient in preventing serious

adverse events like encephalopathy.[186,187] In a recent study, the anti-interleukin (IL)-5 monoclonal antibody reslizumab reduced eosinophilia after treatment with DEC, but did not reduce the incidence of adverse events (which could however be related to low initial microfilaraemia and/or sample size).[188] Finally, it has been speculated that erythropoietin could be useful in preventing treatment-related SAEs, due to its neuroprotective activity—however, data are still lacking.[189]

1.2 Immunomodulation by *Loa loa*

Through millennia of co-evolution with their hosts, helminths have developed potent mechanisms of immune modulation to evade effective immune responses and establish chronic infection in their host.[190,191] Understanding these mechanisms is not only important regarding the pathogenesis and treatment of helminth infections: helminth-modulated immune responses can increase susceptibility to other infectious diseases,[192–194] restrain effectiveness of vaccines[195–198] and influence states of pathological immune activation as allergy and auto-immunity.[199–202] In *Loa loa* infection, many important features of the disease are immune-mediated and remain poorly understood:

1. Chronicity of infection: long-lasting infection with relatively mild symptoms implies potent mechanisms of immune modulation.

2. Microfilarial status: differences in clinical presentation between amicrofilaraemic and microfilaraemic individuals are associated with distinct immunological profiles.

3. Severe adverse events upon treatment: SAEs upon treatment in highly microfilaraemic individuals are linked to a hyper-inflammatory response.

Unraveling the immunological mechanisms underlying these features of chronic loiasis is crucial for improving our understanding of its pathogenesis, prevention and clinical management.

1.2.1 Immunosuppression in chronic infection

Patent helminth infections have been associated with an initial shift towards a pro-inflammatory Th2-type immune response, characterized by the activation of Type 2 helper T cells, cytokines such as IL-4, IL-5, and IL-13, the production of immunoglobulin E (IgE) and peripheral blood eosinophilia.[203] However, establishment of long-term infection correlates with the expansion of regulatory responses, dominated by IL-10 and transforming growth factor beta (TGF-β), that are associated with little pathology and high parasite load.[204–206] These modulator responses affect various agents of the host's immune system:

Interleukin-10- and TGF-β-producing T regulatory cells (Tregs) expand in chronic helminth infections and limit the induction of a protective immune response by suppressing effector T cell differentiation and proliferation.[207–211] Conversely, low Treg levels seem to be crucial for parasite clearance.[212,213] Helminth-induced Tregs cause B cells to produce IgG4, an anti-inflammatory IgG subclass with little complement activation and the capacity to prevent IgE-dependent effector cell degranulation.[214–216] Regulatory B cells induce Tregs through IL-10 and suppress effector T cell cytokine responses (Interferon-γ, IL-4, IL-17).[217] Immunoglobulin G4, IL-4, IL-13 and TGF-β induce alternatively activated macrophages with impaired phagocytic activity, that suppress effector T cell responses through IL-10, TGF-β and PD-L1.[218–223] Finally, helminth infections can impair differentiation and migration of dendritic cells and modulate their function towards regulatory pathways.[224–227]

Although chronic loiasis appears to cause more clinical manifestations than previously recognised, it arguably still causes less (inflammatory) symptoms than might be expected given the subcutaneous migration of foreign-bodies and high levels of circulating microorganisms. So far, only few studies have addressed immunoregulatory responses in loiasis: Chronic *Loa loa* infection—just as other helminth infections—seems to be associated with an increase in Th2-type immune responses with elevated levels of IL-4, IL-5 and IL-13 upon antigen stimulation and elevated IgE.[228–230] At the same time, increased levels of IL-10 upon stimulation *in vitro* imply an expansion of regulatory responses.[230] Indeed, animal models have shown that initial Th1- and Th2-type immune responses—which might be necessary for protective immunity—are downregulated upon patency, leading to a state of T cell hyporesponsiveness.[231–235] In the human host, *in vitro* PBMC proliferation upon *Loa loa*-specific and -unspecific antigen stimulation was shown to be decreased in high- compared to low-transmission settings.[236] *Loa loa* infection has been associated with impaired CD4+ T memory cell responses to tuberculosis antigen, increased risk of other filarial and human T-cell lymphotropic virus type 1 (HTLV-1) infections and may contribute to increased immunopathology in HIV-infected individuals.[228,235,237–239] Infection during pregnancy might lead to increased activation of Tregs in the newborn, suppressing both Th1- and Th17-type immune responses.[240] Besides IgE, parasite-specific IgG4 is elevated in *Loa loa*-infection, suggesting another mechanism of immune modulation.[124]

1.2.2 Immunological differences in microfilarial status

Immune-mediated differences in the clinical presentation of loiasis have first been observed between endemic and transient residents of areas with *Loa loa* transmission presenting at European and North American travel clinics: endemic residents have a higher frequency of microfilaraemia and eye worm and higher microfilaraemia, total

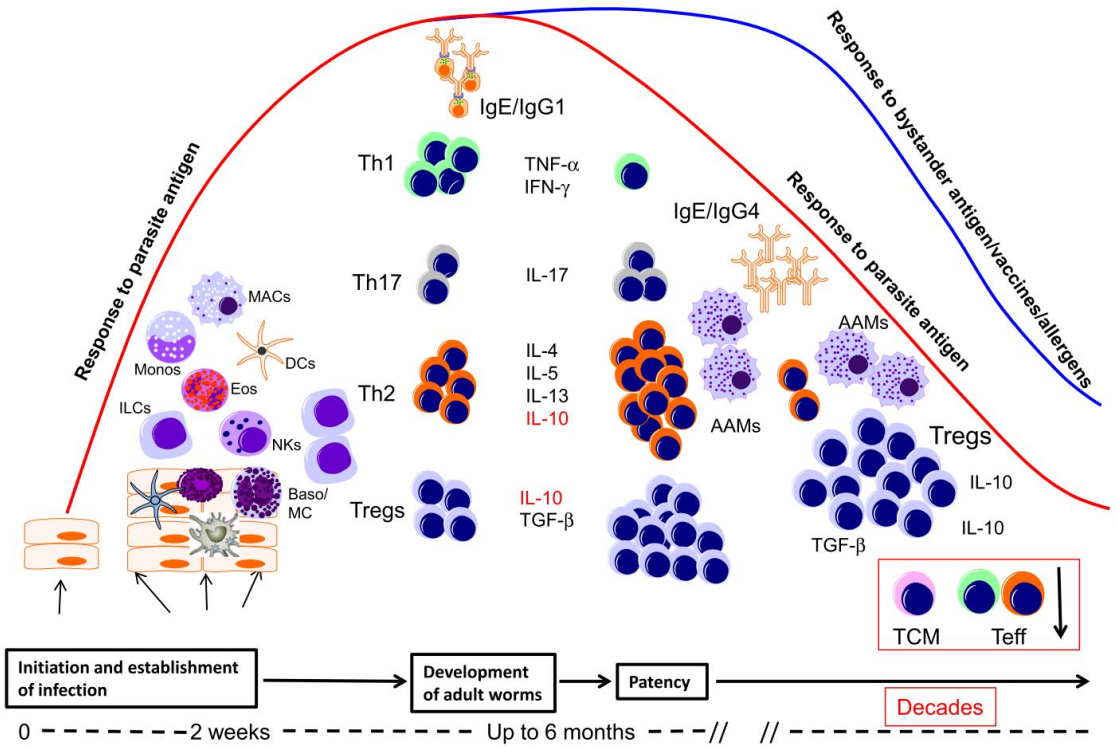

Figure 1.4: Immune responses in helminth infections
"Infectious stages of helminth parasites initiate infection at barrier sites and activate a variety of different cell types such as innate lymphoid cells (ILCs), macrophages (MAC), dendritic cells, natural killer cells (NK), eosinophils (Eos) and basophils/mast cells (Baso/MC). At this relatively early phase of infection, the parasite induces the differentiation of effector Th1, Th17 and Th2 cells, which together with IgE antibody, may lead to the attrition of some of the parasites. At the time of patency (when egg laying/microfilarial release occurs), there is a small expansion of Th2 CD4+ cells and a concomitant contraction of Th1 cells. With the evolution of chronic longstanding infection, there is an associated expansion of IL-10- and/ or TGF-β-producing T regulatory cells (Tregs). The high levels of IL-10 produced induce the production of IgG4, which together with IL-4, IL-13 and/or TGF-b induce the differentiation of alternatively activated macrophages (AAM) and inhibit the function of a variety of other cells including central memory (TCM) and effector (Teff) T cells." Adapted from Nutman.[203]

IgG, and total IgE; while transient residents have higher frequency of Calabar swellings, urticaria and eosinophilia and higher total leukocytes, leukocyte proliferation to parasite antigen and filarial-specific IgG—suggesting states of more pronounced immunotolerance and inflammation associated with tolerance of and protection against microfilariae, respectively.[91,95,97–99,241–244] However, transient residents might also present to travel clinics earlier upon developing symptoms, which means that the differences described above may be related to different phases of the infection and immune responses thereof. Finally, transient residents—through lower level of exposure—may be infected by fewer adult worms, potentially decreasing the odds of successful mating and thus reducing the likelihood of microfilaraemia.

Several studies found that microfilaraemic individuals have decreased *in vivo* levels

of IL-4, IL-5, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) compared to amicrofilaraemic individuals, suggesting an impaired Th2-type and innate immune response.[170,241] Upon antigen stimulation *in vitro*, microfilaraemic individuals additionally had reduced lymphoproliferation and Th1-type immune responses.[245] These aberrations may be linked to increased regulatory responses in microfilaraemic individuals, who display a higher *in vivo* frequency of IL-10 producing cells and increased production of the IL-10 superfamily cytokines IL-19 and IL-24 upon filarial antigen stimulation than amicrofilaraemic individuals.[230,233,246]

The humoral immune response—which has been investigated in endemic areas—also differs depending on microfilarial status: Microfilaraemic individuals have lower levels of filarial-specific IgG compared to amicrofilaraemic individuals.[123,170,245] Moreover, there is an anti-inflammatory shift in IgG subtype expression in microfilaraemic individuals, who produce less IgG1 and more IgG4 than their amicrofilaraemic counterparts.[170,247,248] Finally, microfilaraemic, but not amicrofilaraemic or uninfected individuals, have decreased levels of reactive antibodies to certain *Loa loa* adult and microfilarial surface antigens.[249–256] Whether these differences in antibody levels are caused by increased antibody turnover due to abundant antigens in (microfilaraemic) infected individuals or whether microfilarial status could depend on immunological differences in microfilarial antigen recognition remains to be elucidated.

1.2.3 Inflammatory pathways during treatment

As described above, similar SAEs—with frequency, intensity and timing correlating to treatment-induced decrease in *Loa loa* microfilaraemia—have been observed in individuals treated with DEC, ivermectin and albendazole, suggesting a common pathophysiology.[148] Blood-borne helminth parasites have developed an array of sophisticated strategies to evade activation of the complement system and coagulation cascade, e.g. by disguising with host self-markers, masking their pathogen-associated molecular patterns (PAMPs) and acquiring/producing host complement regulators and anticoagulants.[257] Specifically, *Loa loa* microfilariae carry the host complement regulators factor H and C4b-binding protein to block complement activation.[258] These functions are disturbed upon anthelminthic treatment: Autopsies in humans and baboons have shown that degenerating microfilariae accumulate in small vessels, which leads to fibrin clotting and endothelial damage, initiating hemorrhages and obstructive inflammatory processes—associated with the immigration of eosinophils and macrophages—most prominently in highly vascularized organs such as the brain (encephalopathy) and kidneys (hematuria, proteinuria).[24,259–262] Moreover, microfilariae seem to actively flee from high drug concentrations in the blood circulation, penetrating tissue and entering

cerebrospinal (CSF), pleural, peritoneal/-cardiac fluids and urine.[24,172,262] Microfilarial tissue penetration is associated with accumulation of inflammatory cells—especially eosinophils—and necrosis in various organs including the brain and may lead to formation of granulomas.[186,259,262,263] Based on these pathological findings, it was hypothesized that a hyper-inflammatory response with massive tissue migration of eosinophils and release of toxic mediators dominates the pathogenesis of treatment-related SAEs in loiasis.[262]

Indeed, immunological studies in humans and primates revealed that treatment with DEC or ivermectin is associated with increased general inflammatory (C-reactive protein (CRP), neutrophils) and Th2-type (eosinophils, IL-5, IL-9, IL-10, IL-13) immune responses.[24,148,262,264] Herrick at al. found that specifically peak Th2-type cytokine levels (IL-5, IL-9, IL-10, IL-13) after treatment with DEC or ivermectin correlated with baseline microfilaraemia.[148] In their study, absolute eosinophil counts temporarily decreased upon treatment with DEC and increased upon treatment with ivermectin in the first 24 hours after intake; followed by an increase in both groups which peaked between day 5 and 9. Adverse event (AE) incidence peaked eight and 24 hours after intake of DEC and ivermectin, respectively. The drop in eosinophil counts post-DEC and increases in IL-8 and eotaxin-1 (both inducing chemotaxis in eosinophils) suggest rapid tissue migration of eosinophils. Moreover, eosinophils showed an increase in surface expression of the activation marker CD69 upon treatment. Together, the correlation of baseline microfilaraemia with Th2-cytokines and the consistent pattern of faster microfilarial clearance, followed by earlier peaks of AE frequency, IL-5 levels and CD69 expression in patients treated with DEC compared to ivermectin underlines the close relationship between microfilaraemia, IL-5 induced eosinophilia and AEs upon treatment of loiasis.

1.2.4 Eosinophils

Eosinophils are predominantly tissue-dwelling white blood cells of the granulocyte lineage. Eosinophilia has long been identified as a hallmark of helminth infection and eosinophil's capacity *in vitro* to kill helminth larvae has established their role as protective effector cells at the end of a type 2 immune response.[265] However, *in vivo* investigations in the murine model over the last decades revealed a more multifaceted role of eosinophils in helminth infections, which nevertheless remains incompletely understood.[266]

Eosinophils develop in the bone marrow under the influence of IL-3, IL-5 and GM-CSF.[267,268] They make up around 1–5% of peripheral blood leukocytes and spend only 8–18 hours in the circulation before they migrate to tissue, where they are a hundred times more abundant.[269] In homeostasis, tissue migration is mostly stimulated by eotaxins.[270] In inflammatory sites, activated Th-2 lymphocytes produce IL-4, IL-5

and IL-13, that induce the release of eotaxins and RANTES (regulated on activation, normal T cell expressed and secreted).[271] IL-5, eotaxins and RANTES both attract and activate eosinophils by binding to the IL-5 receptor α (also designated CD125; binding IL-5), C-C chemokine receptor type 3 (CCR3/CD193; eotaxins, RANTES) and CX3C motif chemokine receptor 1 (CX3CR1; eotaxins).[272–275] Activated eosinophils release degranulation proteins, such as MBP (major basic protein), EPO (eosinophil peroxidase), EDN (eosinophil-derived neurotoxin), and ECP (eosinophil cationic protein), that cause parasite cytotoxicity and tissue damage.[276] However, they are also capable of antigen-presentation and inducing type 2 and other immune responses through release of cytokines (IL-2, IL-4, IL-5, IL-6, IL-13, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), GM-CSF, eotaxin, RANTES) and direct interactions with CD4+ T cells, dendritic cells, B cells and mast cells.[271] Phenotypically, eosinophil activation is associated with downregulation of CD125 and CD193 surface expression in a negative feedback loop, and upregulation of CX3CR1.[235,277–280] Moreover, activated eosinophils express the early lymphocyte activation marker CD69, which is induced by IL-3, IL-5 and GM-CSF.[281] Eosinophil progenitors and young eosinophils express higher levels of the IL-3 receptor α (CD123).[282] Finally, the IgG-receptor FcγRIII (CD16) is upregulated in eosinophils upon stimulation with various cytokines and in some states of allergy.[283,284]

As described above, eosinophils might play a vital role in both differences in clinical presentation of *Loa loa* as well as treatment-related SAEs. Moreover, given that chronic eosinophilia can cause immunopathologies such as endomyocardial fibrosis—which has been described as a reason for heart failure in patients with loiasis—one can speculate that eosinophils may play a role in long-term morbidity and mortality.[27,285] Thus, insights into eosinophil activation and expression patterns of surface activation markers in this context can contribute to a better understanding of immunomodulation by *Loa loa*.

1.2.5 Basophils

Basophils are the rarest subpopulation of granulocytes, predominantly found in the circulation. Traditionally associated with allergy and anaphylaxis, their role in the immune response to helminth parasites—primarily through initiation or amplification of type 2 immune responses—has become evident.[286]

Basophils develop in the bone marrow under the regulation of IL-3 and other factors such as thymic stromal lymphopoietin (TSLP). Basophils constitute around 0.5% of blood leukocytes.[287,288] While they may migrate increasingly to sites of inflammation, peripheral blood basophilia is rare in parasitic infections.[289] Basophils are activated by complement factors, cytokines (IL-3, GM-CSF, TSLP) and helminth-derived antigens through IgE cross-linking and Toll-like receptor ligands.[290] Upon activation they release

tissue-active inflammatory factors such as histamine, leukotrienes and prostaglandins that are responsible for the symptoms observed in allergic reactions.[291] Beyond these effector functions, basophils release cytokines such as IL-4, IL-13, IL-6 and TNF-α.[290] Basophils secrete IL-4 upon stimulation by helminth antigen in both helminth-exposed and -naive individuals—earlier and in greater quantity than CD4+ T cells—implying a key role in initiating and amplifying type 2 immune responses.[292–295] Moreover they can present antigen, initiate IgE isotype-switching in B cells and induce the development of alternatively activated macrophages.[296–298]

Chronic helminth infection seems to suppress the responsiveness of basophils, which can cause allergic reactions and are key players in initiating type 2 immune responses against parasites.[299,300] However, neither basophils nor their phenotypic activation have ever been investigated in loiasis.

1.2.6 Myeloid-derived suppressor cells (MDSC)

MDSC are a heterogenous group of immature myeloid cells with potent immunoregulatory capacities, that have been extensively described in cancer. However, MDSC also play a key role in modulating immune responses in infectious diseases and helminth infection.

There are two MDSC subgroups, polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) and monocytic myeloid-derived suppressor cells (M-MDSC), with morphological similarities to neutrophils and monocytes, respectively. Physiologically, MDSC constitute around 0.5% of PBMC.[301] They expand in bacterial, viral, fungal and parasitic infections induced by factors such as vascular endothelial growth factor (VEGF), GM-CSF, granulocyte colony-stimulating factor (G-CSF), IL-1β, IL-6, TNF-α, prostaglandin E2 (PGE2), toll-like receptor ligands and certain helminth products.[302,303] MDSC impair T cell responses through suppressive factors such as NADPH oxidase, reactive oxygen species (ROS) and peroxynitrite (PMN-MDSC); and inducible nitric oxide synthase (iNOS) and nitric oxide (NO) (M-MDSC).[304] They downregulate the T cell receptor through Arginase-1, secrete IL-10 and TGF-β, inhibit B cell responses and impair maturation of dendritic cells and natural killer cells.[305,306] Through surface expression of PD-L1, activated MDSC reduce proliferation and induce apoptosis of T effector cells, while promoting T regulatory cells.[307–309]

MDSC have been shown to expand not only in various parasitic diseases,[310–317] but also in mouse models of nematode and filarial infections[318–320]—suggesting a possible role of MDSC-mediated immunosuppression in chronic *Loa loa* infection.[235] However, MDSC subsets and their expression of PD-L1 have never been investigated in loiasis.

1.3 Research questions

In resumé, the three key features of loiasis described above—chronicity of infection, differences in microfilarial status and severe adverse events upon treatment of highly microfilaraemic individuals—are considered to be immune-mediated but remain poorly understood. Eosinophils, and likely also basophils and MDSC, appear to play a central role in these processes, although to our knowledge, the latter two have never previously been investigated in loiasis.

Here, we aimed to study these leukocyte populations and their surface activation, as well as associated circulating cytokine levels both in a setting of chronic microfilaraemic and amicrofilaraemic infection, and during perturbations caused by treatment. To this end, we assessed these immunological parameters in samples collected in the context of two human studies: a cross-sectional study including *Loa loa*-uninfected, -amicrofilaraemic and -microfilaraemic individuals and a longitudinal study including microfilaraemic individuals undergoing treatment. Our aim was to test the following specific hypotheses:

1. Is chronic *Loa loa* infection associated with regulatory responses, i.e. i) expansion of MDSC subset numbers and their PD-L1 expression, ii) decrease in numbers and phenotypic activation of eosinophils and basophils, and iii) a shift from associated proinflammatory (IL-1, IL-4, IL-5, IL-6, IFN-γ, TNF-α, GM-CSF) towards anti-inflammatory (IL-10) cytokine profiles?

2. Is the presence versus absence of microfilaraemia in *Loa loa*-infected individuals associated with even further expansion of these immunoregulatory responses (1.i–iii) compared to amicrofilaraemic infected individuals?

3. Does treatment of *Loa loa*-microfilaraemic individuals with albendazole lead to an inflammatory response in terms of i) increase in numbers and phenotypic activation of eosinophils and basophils, ii) increase of associated proinflammatory cytokines (IL-1, IL-4, IL-5, IL-6, IFN-γ, TNF-α, GM-CSF), and iii) a co-activation of regulatory MDSC and increased IL-10 levels? Do the immune perturbations observed in *Loa loa*-infected individuals compared to non-infected (1.i–iii) resolve after treatment?

2 Materials and methods

2.1 Study site

The research activities for this thesis were conducted at the Centre de Recherches Médicales de Lambaréné (CERMEL) between March 2018 and February 2020. CERMEL is a leading African medical research center situated in the provincial town of Lambaréné, Gabon in the Central African rain forest region.[321] The upper-middle-income country is sparsely populated by two million people, with one of the highest gross domestic products per capita in the region due to rich oil and manganese resources.[322] However, 30% of the population lives below national poverty limits and Gabon ranks 115 out of 189 in the human development index—186 when related to gross national income per capita.[322,323]

Study participants were screened in the districts of Estuaire, Moyen-Ogooué and Ngounié along a roughly 200-kilometer section of the national route N1—the major, yet partly unsurfaced traffic artery connecting the capital Libreville to the southeastern parts of the country (Figure 2.1). The region is characterized by tropical rain forest and equatorial climate with an average temperature of 26.1°C and humidity above 80%.[324,325] The local population inhabits small, rural villages scattered along the national route and makes a living through farming and hunting.[73] Infectious diseases—malaria, helminth infections and multifactorial syndromes such as pneumonia and diarrhea—are highly endemic, impose a major burden of disease and cause up to 71% of health care consultations.[326–330] At the same time, non-communicable diseases are on the rise.[330,331]

Loiasis is highly endemic in the study area: A national study conducted in 2005 and 2006 found a prevalence of *Loa loa* microfilaraemia between 18.6% and 33.4% in the population of Estuaire, Moyen-Ogooué and Ngounié.[72] A more recent publication estimated the prevalence of *Loa loa* microfilaraemia at 10.7% in Ngounié and a combined (microfilaraemia or eye worm history) prevalence of 30.5%.[332] A recent study from our center found that 50.8% of participants in Tsamba-Magotsi and Ogooué et des Lacs were *Loa loa*-infected.[5] Studies in hospital patients and pregnant women are in accordance with the high prevalence in the general population.[100,326]

All research activities were conducted in accordance with the Declaration of Helsinki, Good Clinical Practice, Good Clinical Laboratory Practice and local regulations.[333–335] The project was approved by the Institutional Ethics Committee at Centre de Recherches Médicales de Lambaréné (reference CEI-022/2018), as were the underlying cross-sectional (CEI-011/2017) and treatment studies (CEI-013/2017).

Figure 2.1: Study site
The study was conducted in the districts of Estuaire, Moyen-Ogooué and Ngounié, Gabon in small, rural villages along the national route N1 (left photograph showing an example of a village selected for screening at −0.38640, 10.34701). The study center and laboratory were located at the Centre de Recherches Médicales de Lambaréné (CERMEL) in the provincial town of Lambaréné (right). Photograph by Gerrit Burger, maps adapted from openstreetmap.org.[336]

2.2 Study design

Immunological samples from two human studies were analyzed: a cross-sectional case-control study to compare immunomodulation among *Loa loa*-infected amicrofilaraemic and microfilaraemic participants with uninfected controls; and a randomized controlled open-label study to assess inflammatory responses in microfilaraemic patients undergoing different treatment regimens with albendazole and ivermectin. To recruit participants, a screening for *Loa loa*-infected individuals was conducted. Figure 2.2 outlines the study designs and sampling for immunological analyses.

2.2.1 Screening

Rural villages in the study area were selected for screening in a practical manner. Residents aged at least two years were invited to participate. A study team member visited the selected village prior to screening to discuss the study purposes and procedures with the local community, represented by the village leader (chef·fe de village). Screenings were only conducted with their approval and invitation. The study's background, purposes, procedures, risks and benefits were explained to every participant. Written informed consent—for screening and a potential second blood collection for immunological analyses—was obtained. In participants below the age of 18, a legal representative was required to sign the consent form in addition to the minor's assent. Illiterate or non-

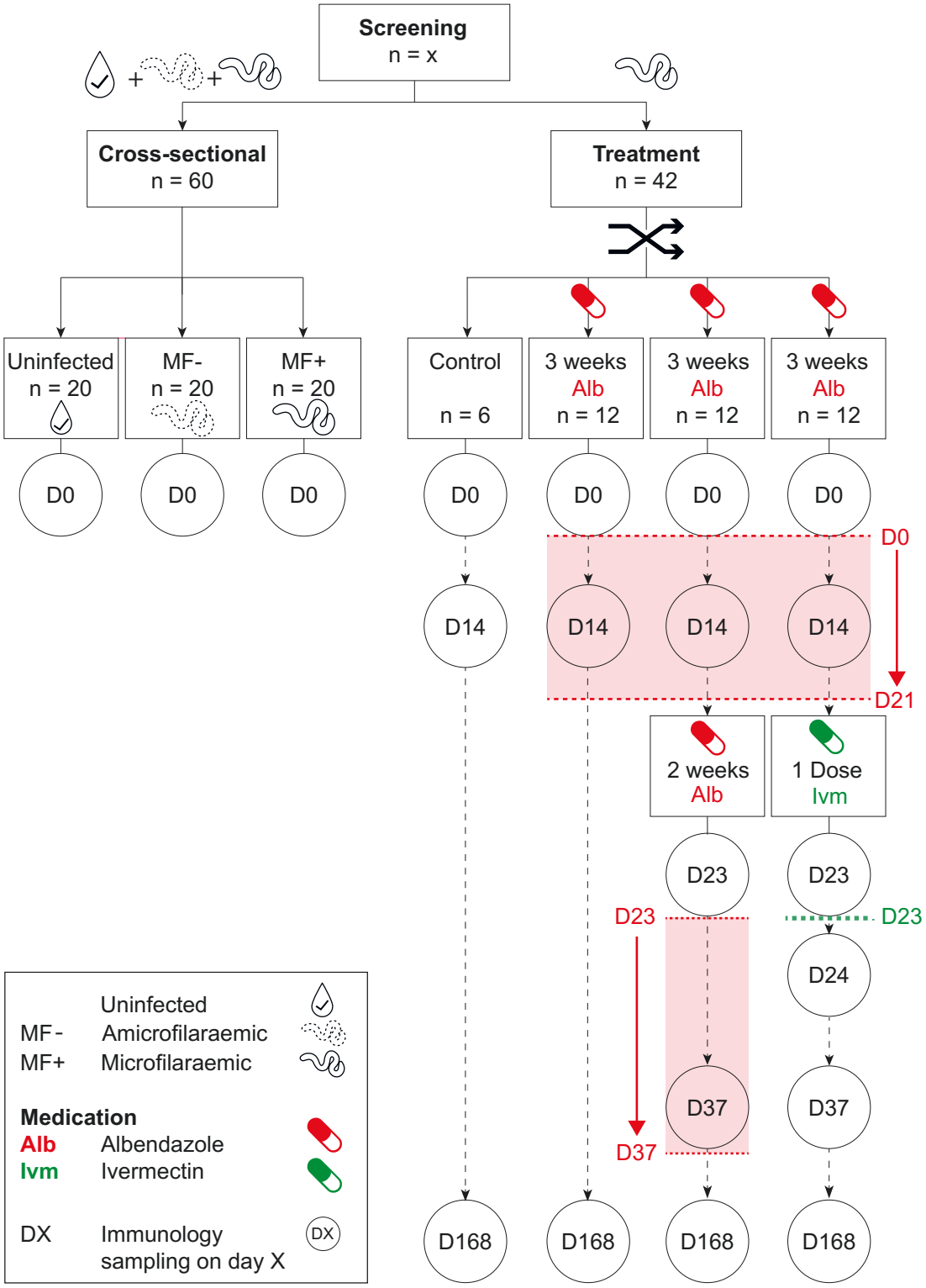

Figure 2.2: Study design and sampling for immunology
Participants were screened for inclusion into the cross-sectional study (left) and randomized controlled (treatment) trial (right). They were classified into a parasitologically defined group (uninfected, amicrofilaraemic, microfilaraemic) or randomized to a treatment arm (control, 3 weeks albendazole, 3 + 2 weeks albendazole, 3 weeks albendazole + single-dose ivermectin), respectively. Samples for immunological analyses were collected at baseline and various time points of treatment until end of follow-up at six months (day 168).

Table 2.1: Inclusion criteria for the cross-sectional case-control study

	Uninfected	Amicrofilaraemic	Microfilaraemic
History of eye worm	−	+*	
Loa loa microscopy	−	−	+
Loa loa serology	−	+	

− negative, + positive
* if possible recent, participants < 15 years were included disregarding history of eye worm

francophone participants were supported in the consent procedure by a witness independent from the study team (e.g. a literate family member fluent in the local language).

To assess the history of eye worm, the rapid assessment procedure for *Loa loa* (RAPLOA) questionnaire was administered (Section 2.3.1). The last episode of eye worm was documented in a subset of participants. A malaria rapid diagnostic test (RDT, Paracheck Pf, Orchid Biomedical Systems, Goa, India) was performed according to the manufacturer's specifications and participants with positive results were offered immediate antimalarial treatment.

A trained nurse collected up to 7.5 ml venous blood in ethylenediaminetetraacetic acid (EDTA) tubes for *Loa loa* microscopy (2.3.2) and serology (2.3.3). All blood collections were performed between 10:00 and 15:00 due to diurnal periodicity of *Loa loa* microfilaraemia. Blood samples were transported to the laboratory in a cool and dark container. Next, 300 μl of EDTA-anticoagulated blood was transferred to a 1.5 ml Eppendorf tube and centrifuged (10 minutes (min), 4000 rounds per minute (rpm)). For serologic testing (2.3.3), 100 μl of the plasma supernatant was stored at -80°C in a 1.5 ml Eppendorf tube.

2.2.2 Cross-sectional case-control study

The cross-sectional case-control study (ethics reference CEI-011/2017) aimed to include 60 residents of the study area with an age of at least two years in three groups related to infection status: *Loa loa*-uninfected; *Loa loa*-infected and amicrofilaraemic; and *Loa loa*-infected and microfilaraemic (Table 2.1). Preliminary classification of screened participants was based on RAPLOA and microscopy results; uninfected and amicrofilaraemic status were serologically confirmed in participants considered for inclusion. Malaria-infected individuals—whose immune responses can be heavily skewed—were excluded from the study.[337] Participants were enrolled equally across age cohorts < 15, 15–40 and > 40 years matched across infection groups by sex, age and, as far as possible, village and date of screening. Venous blood was collected once for immunological analysis (Section 2.4.1).

Table 2.2: Inclusion and exclusion criteria for the randomized controlled trial

Inclusion criteria	Exclusion criteria
Resident of the study area	Intolerance to study drugs
Age $\geq$ 18 years	Albendazole intake < 4 weeks before inclusion
Loa loa microfilaraemia 5,000–50,000 mf/ml	Active liver disease (e.g. HBV, HCV)
Written informed consent	Immunosuppressive condition (e.g. HIV)
	History of epilepsy, encephalitis, meningitis or encephalopathy
	Pregnancy, breastfeeding or premenopausal women not using contraception

HBV: hepatitis B virus, HCV: hepatitis C virus, HIV: human immunodeficiency virus

2.2.3 Randomized controlled trial

The randomized controlled open-label trial (ethics reference CEI-013/2017) aimed to include 42 adult residents of the study area with a *Loa loa* microfilaraemia below the safety threshold of 50,000 mf/ml and not meeting any exclusion criteria (Table 2.2).[338] Potential participants were mostly recruited from screening but also from other studies conducted at the research center. Informed written consent was obtained after explanation of the study's background, purposes, procedures, risks and benefits. Blocks of seven participants were randomized to the four study arms in a ratio of 1:2:2:2 in a continuous manner throughout the study period. Arm 1 and 2 as well as arm 3 and 4 of each randomization block started treatment in parallel. Individuals who refused further participation in the study at any point after randomization were replaced based on a sequential list with eligible participants.

Figure 2.2 shows treatment procedures and time points for immunology sampling in each study arm. Participants in arm 1 received no anthelminthic treatment (control group). Arm 2 received a three-week course of albendazole (2 x 400 mg/d, Medopharm, Chennai, India) and arm 3 an additional two-week course of albendazole beginning on day 23. Participants in arm 4 received a three-week course of albendazole followed by a single dose of ivermectin (150 μg/kg, MSD, Kenilworth, USA) on day 23 if microfilaraemia had dropped below 4,000 mf/ml, the predefined security threshold for ivermectin treatment. Else, they received another two-week course of albendazole. Symptomatic treatment (i.e. loratadine for pruritus or paracetamol for arthralgia) was offered in all study arms if clinically indicated. Participants receiving albendazole were advised to take in the drug with a meal to ensure adequate bioavailability. Participants in the control group were offered a four-week course of albendazole (2 x 400 mg/d) at the end of follow-up.

Study visits were performed at baseline, twice a week in the first three weeks of any

Table 2.3: RAPLOA questionnaire with answers defining history of eye worm

Item	Answer
Have you ever experienced or noticed worms moving along the white part of your eye?	Yes
Have you ever had the condition in this picture?*	Yes
The last time you had this condition, how long did the worm stay before disappearing?	$\leq$ 7 days

* asked whilst showing a photograph of an adult *Loa loa* worm in the eye

treatment (first or second course), weekly until day 56 and twice a month until day 168. On each visit, venous blood for *Loa loa* microscopy (Section 2.3.2) was collected in an 1.2–7.5 ml EDTA tube and patients were surveyed for adverse events. Sampling for immunological analyses (Section 2.4.1) was performed at various time points (Figure 2.2).

2.3 *Loa loa* diagnostics

2.3.1 Rapid assessment procedure for *Loa loa* (RAPLOA)

To assess the history of eye worm, the RAPLOA questionnaire was administered in accordance to WHO guidelines.[339] The test was originally developed and validated to assess prevalence and intensity of loiasis at the community level in epidemiological studies.[2,340,341] It is based on three items including confirmation by recognition of an adult *Loa loa* worm in the eye on a photograph (Table 2.3).

2.3.2 Microscopy for *Loa loa* microfilariae

Microscopy for *Loa loa* microfilariae was performed either directly on TBS or after leukoconcentration, as described previously.[5] Since the latter uses a higher blood volume and thus has a significantly higher sensitivity, additional leukoconcentration was performed for negative TBS from eligible participants. See Tables S1–S3 for materials used.

TBS were prepared in duplicates according to the Lambaréné method.[343] Briefly, 10 μl of EDTA-anticoagulated blood was evenly spread in a 10 x 18 mm rectangle on a microscopy slide with a micropipette and air-dried. TBS were stained with Giemsa solution (20% Giemsa in phosphate buffer, 20 min), carefully washed in distilled water and air-dried before microscopy. For leucoconcentration, 1 ml of saponin solution (1% saponin in NaCl 0.9%) was added to 1 ml of EDTA-anticoagulated blood in a 15 ml Falcon tube, gently mixed and incubated for 5 min. After centrifugation (5 min, 2000 rpm), the supernatant was carefully discarded and the whole pellet transferred to a microscope

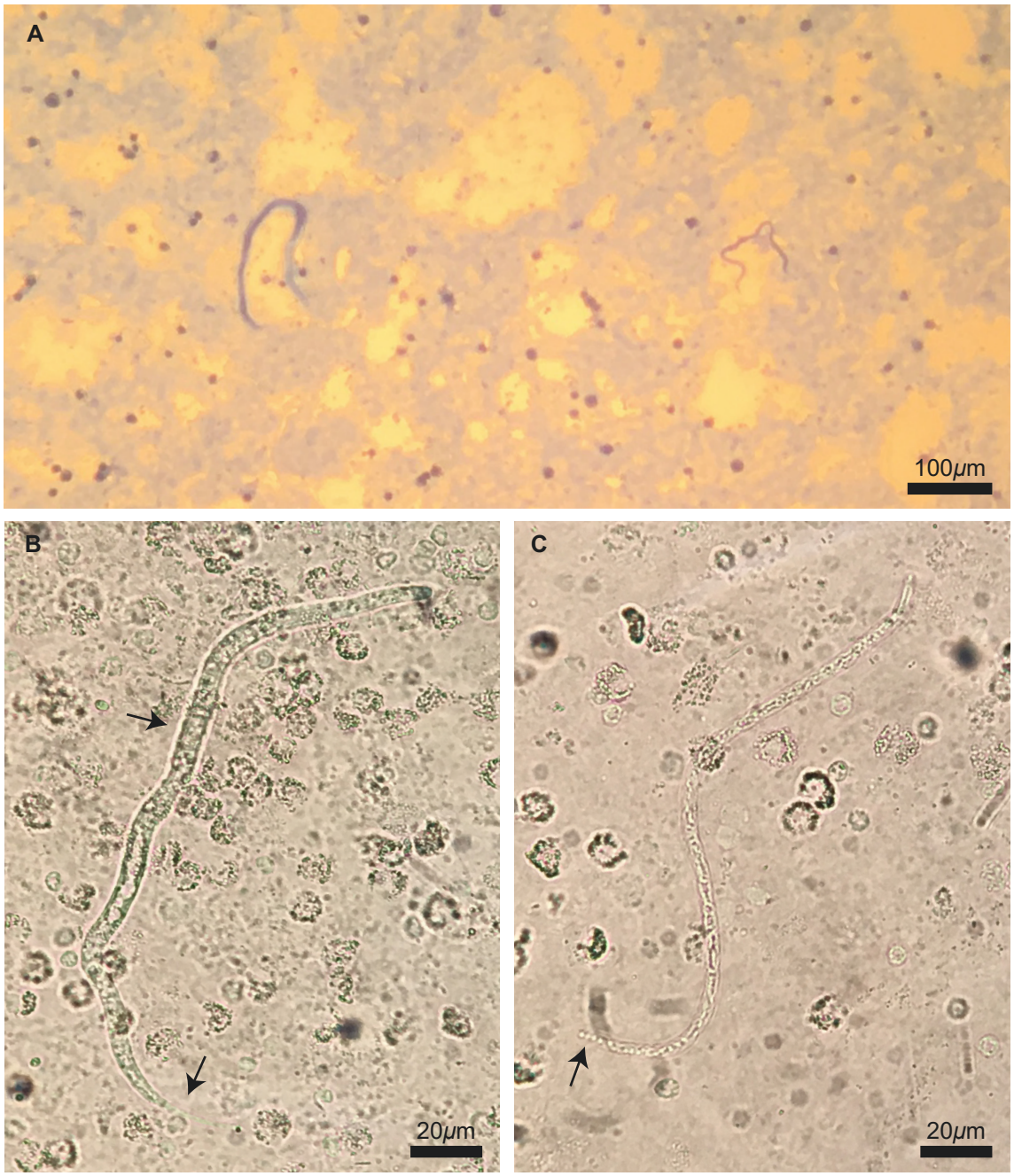

Figure 2.3: Microscopy for *Loa loa* microfilariae
(A) *Loa loa* (left) and *Mansonella perstans* (right) microfilariae, Giemsa-stained thick blood smear, 10x objective. (B) *Loa loa* microfilaria, leucoconcentration technique, 40x objective. Note the typical sheath and tapered tail (arrows). (C) *Mansonella perstans* microfilaria, leucoconcentration technique, 40x objective. Note the smaller size, blunt tail (arrow) and absence of a sheath. Also see video of a live *Loa loa* microfilaria (DOI: 10.6084/m9.figshare.23615034).[342] Photographs and video by Gerrit Burger.

slide using a 1 ml pipette. The pellet was spread out by applying a cover slip with gentle pressure. Microscopy was performed on freshly prepared slides only.

The whole slides were read by trained microscopists on a suitable microscope using the 10x objective. Specific characteristics of microfilariae—such as size, presence of a sheath, and form of the tail (Figure 2.3)—were assessed using the 40x objective to differentiate between *Loa loa* and *Mansonella perstans*, the two common filaria species in the study area.[5] For TBS microscopy, the total number of microfilariae counted in the two blood smears was multiplied by 50 to calculate microfilaraemia per milliliter.

2.3.3 *Loa loa* serology

Serological diagnosis was performed by the *Loa loa* SXP-1 IgG antibody rapid test (Drugs & Diagnostics for Tropical Diseases, San Diego, USA) with a sensitivity of 94% and specificity of 100% for life-time exposure to all parasite stages.[128] The test was performed according to the manufacturer's specifications: it was placed flat on an even surface and 5 μl of thawed EDTA plasma followed by two drops of buffer were transferred to the port. Results were obtained visually after 20 to 60 min and any visible line was considered positive.

2.4 Overview of laboratory procedures

2.4.1 Sampling and workflow

At each immunology time point in either study, venous blood was collected in a 10 ml sodium-heparin tube by a trained nurse and transported to the laboratory at CERMEL in a cool and dark container within approximately two hours. Irregularities (e.g. signs of coagulation or hemolysis) were checked and noted upon arrival. For each sample, full blood was lysed to assess eosinophil/basophil activation by flow cytometry; plasma was isolated and cryopreserved to quantify pro- and anti-inflammatory cytokines by cytometric bead array; and PBMC were isolated to analyze MDSC subsets by flow cytometry. Remaining PBMC were cryopreserved for future assays. A T cell proliferation-suppression assay was performed on a subset of samples to confirm functional immunosuppressive capacity of PMN-MDSC.

Basic laboratory equipment, consumables, media and solutions are listed in Tables S1–S3. Other materials are specified in their respective methods section. If not stated explicitly, procedures were performed at room temperature and laboratory equipment used with default settings.

2.4.2 Plasma isolation

200 μl of whole blood was removed for the eosinophil/basophil panel (Section 2.5.1) before plasma isolation. The remaining blood was transferred to a 15 ml Falcon tube and centrifuged (7 min, 1,500 rpm). Up to 4.5 ml of plasma supernatant was cryopreserved at -80°C in 1.5 ml Eppendorf tubes.

2.4.3 Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Figure 2.4) according to a standard protocol used in the CERMEL laboratory.[344] The remaining volume after plasma isolation was transferred to a 50 ml Falcon tube and diluted to twice the initial blood volume with Hank's balanced salt solution (HBSS) (1% penicillin-streptomycin-glutamine (Pen-Strep-Glu)). The dilution was carefully layered on top of half its volume of Ficoll density gradient medium in another 50 ml Falcon tube. After centrifugation (25 min, 1,500 rpm, lowest acceleration, no brake), the supernatant was discarded and the PBMC layer carefully collected and transferred to a 50 ml Falcon tube with a 10 ml Stripette. Cells were washed twice in 40 ml and 10 ml HBSS, respectively by centrifuging (10 min, 1,500 rpm), discarding the supernatant and resuspending the pellet. 5 ml of complete medium (Roswell Park Memorial Institute medium (RPMI), 10% fetal bovine serum (FBS), 1% Pen-Strep-Glu, 1% Sodium-Pyruvate) was added and 50 μl of the suspension transferred to a 1.5 ml Eppendorf tube for manual cell counting. PBMC in complete medium were diluted 1:10 with Turk's solution and transferred to a hematocytometer (Neubauer's counting chamber). Cells in two diagonal 0.1 mm^3 fields were counted. If necessary, the initial dilution was adapted to reach an optimal cell density (20–50 per field). The count was multiplied by dilution factor and resuspension volume to calculate the total number of isolated PBMC.

2.4.4 PBMC cryopreservation

After removing 5 x 10^5 PBMC for the MDSC panel, remaining cells were cryopreserved in aliquots of 5 x 10^6–10 x 10^6 cells for future assays: PBMC were centrifuged (10 min, 1,500 rpm) and the supernatant was discarded. The pellet was resuspended carefully, and the tube was transferred on ice. 1 ml of pre-cooled freezing medium (RPMI, 20% FBS, 10% dimethyl sulfoxide, 1% Pen-Strep-Glu, 1% Sodium-Pyruvate, 5°C) per aliquot were added dropwise while gently rocking the tube. 1 ml of PBMC in freezing medium were transferred to a 1.8 ml cryotube for each aliquot. Cryotubes were inserted into pre-cooled Mister Frosty freezing containers and stored at -80°C. The samples were transferred to -160°C on the next day for long-term storage.

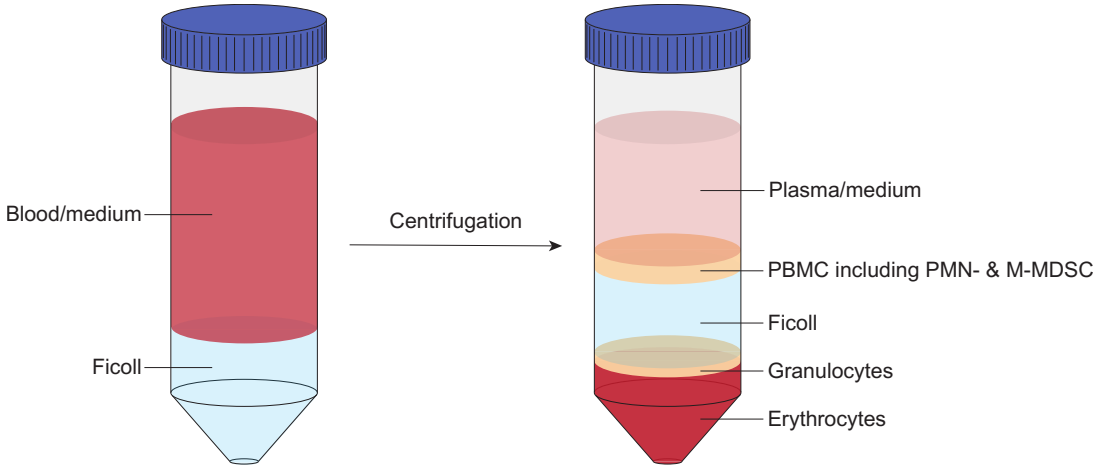

Figure 2.4: Principal of peripheral blood mononuclear cell (PBMC) isolation
Diluted blood is layered over Ficoll density gradient medium and centrifuged. The low-density PBMC layer can be collected above the Ficoll and contains lymphocytes and monocytes—including monocytic myeloid-derived suppressor cells (M-MDSC). Polymorphonuclear MDSC (PMN-MDSC)—despite sharing many phenotypic properties of (high-density) neutrophils—are low-density granulocytic cells also located in the PBMC layer.[345]

2.5 Immunological assays

2.5.1 Eosinophil and basophil flow cytometry panel

For the analysis of eosinophil and basophil numbers and activation state, whole blood was incubated with a mixture of antibody-bound fluorochromes (Table S4). After red blood cell lysis and washing, percentage of eosinophils (defined as side scatter $(SSC)^{hi}$ $CD125^{+}$ $CD193^{+}$), basophils (SSC^{lo} CD193+ CD123+) and expression levels of activation markers (CD16, CD69, CD123, CX3C motif chemokine receptor 1 (CX3CR1)) were analyzed by flow cytometry.

Assay development

All antibody candidates for the final panel were titrated to optimize contrast between stained and unstained cell populations. For this, 100 μl of sodium heparin anti-coagulated blood was incubated with a series of different antibody volumes, starting with the manufacturer's recommendation and stepwise halving volume until it was below 1 μl. After incubation, erythrocyte lysis, washing and acquisition (see final protocol), single granulocytes were gated and investigated for expression of the respective marker in software analysis. Positive and negative population were defined, and contrast quantified by separation index (SI):[346,347]

$$SI = \frac{MFI_{pos} - MFI_{neg}}{P_{84}FI_{neg} - MFI_{neg}} \times 0.995 \quad (1)$$

where MFI_{pos} : median fluorescence intensity of positive population

MFI_{neg} : median fluorescence intensity of negative population

$P_{84}FI_{neg}$: 84^{th} percentile fluorescence intensity of negative population

SI was plotted against antibody volume, and the volume at the maximum SI was selected for further use (Figure S1).

Two solutions for erythrocyte lysis were evaluated: an ammonium chloride solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA) and fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences, San Jose, USA). FACS lysing solution shows a better discrimination of major cell populations (lymphocytes, monocytes, granulocytes) in the scatter plot,[348] but little is known about specific effects on eosinophils. Thus, 100 μl of sodium heparin anti-coagulated blood was incubated with CD3 PE, CD193 PerCP-Cy5.5, Siglec-8 PE-Cy7 (all BioLegend, San Diego, USA), CD14 APC-Cy7 and CD66b FITC (both BD Biosciences, San Jose, USA) and 2 ml of either ammonium chloride or FACS lysing solution added. After incubation, washing and acquisition (see final protocol), single cells were gated and major cell populations as well as CD66b+CD193+Siglec-8+ eosinophils were investigated for their scatter properties. In direct comparison we observed better discrimination of major cell populations—and specifically eosinophils—using the FACS lysing solution (Figure S2), which was used in all further experiments.

Among the candidate antibodies, three eosinophil markers were evaluated: CD125, CD193 and Siglec-8. Since 98% of CD125+CD193+ granulocytes expressed Siglec-8 in initial experiments and limited fluorescent channels (n = 6) were available, we defined eosinophils on CD125 and CD193 only. This allowed us to include CD123, another eosinophil activation marker, in the final panel.

Protocol

100 μl of sodium heparin anti-coagulated blood was transferred to each of two 5 ml Falcon tubes. 6.5 μl of freshly prepared eosinophil/basophil antibody master mix (Table S4) was added to one tube (stained) whilst the other one served as a control (unstained). Both tubes were vortexed and incubated for 15 min in the dark. 2 ml of FACS lysing solution (BD Biosciences, San Jose, California) was added to each tube. The dilution was mixed and incubated for 10 min in the dark. After centrifugation (5 min, 2 000 rpm), the supernatant was decanted, and the pellet resuspended by vortexing. Samples were washed twice by adding 2 ml phosphate-buffered saline (PBS), centrifuging (5 min, 2 000 rpm)

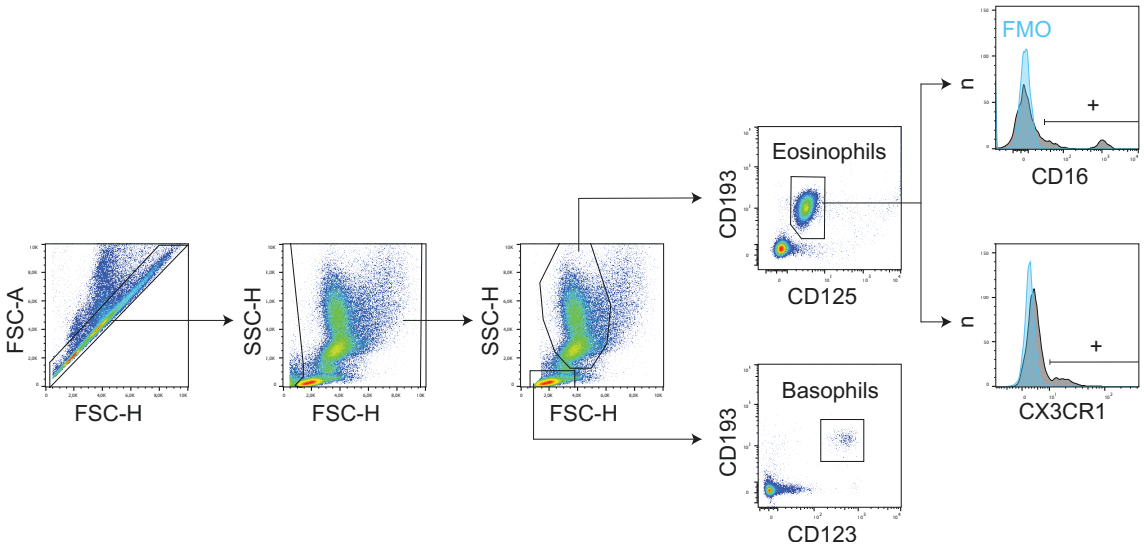

Figure 2.5: Gating strategy for eosinophils and basophils
Eosinophils were defined as SSC^{hi} CD125+ CD193+ single leukocytes; basophils as SSC^{lo} CD193+ CD123+. Expression of activation markers was either directly calculated by MFI or by defining the percentage of cells expressing the marker—e.g. of eosinophils expressing CD16. Unstained and fluorescence minus one (FMO) controls were considered to set gates objectively. Adapted from Burger et al.[235]

and decanting the supernatant. The residuals were transferred to 1.5 ml Eppendorf cups prefilled with 200 μl PBS and mixed by pipetting up and down right before acquisition on a Guava easyCyte 8HT flow cytometer (Merck Millipore, Burlington, USA).

Compensation controls were performed weekly: One drop of each positive and negative control beads (compensation particles set, BD Biosciences) was added to six 5 ml Falcon tubes and each tube was stained with one of the master mix antibodies. Because of the bead's specificity against mouse antibodies, CX3CR1 APC (rat IgG2b) had to be replaced by CD25 APC (mouse IgG1, BD Biosciences) for compensation. Tubes for compensation underwent the same protocol as above, only that PBS was used instead of FACS lysing solution and Eppendorf cups for acquisition were prefilled with 100 μl PBS.

Fluorescence minus one (FMO) controls were performed once for every antibody in the panel: 100 μl sodium heparin anti-coagulated blood from a healthy volunteer was incubated with five of the six antibodies used in the panel (i.e. the FITC FMO control was stained with all antibodies in the master mix except CD69 FITC). The controls were then processed and acquired according to the regular protocol.

For acquisition, the Guava easyCyte 8HT flow cytometer was started up according to the manufacturer's recommendations, including a cleaning program and quality check (Guava ICF cleaning fluid and Easy Check Kit, Merck Millipore, Burlington, USA). Any quality problems—e.g. due to a deceased ant blocking the flow cell—were solved before initiating measurements. Guava InCyte software (Merck Millipore) was used to acquire

the maximal number of events using predefined gain, threshold and flow rate settings. After acquisition, data were exported as flow cytometry standard (FCS) files for data analysis.

Data analysis

FCS files were imported into FlowJo software version 10.6.1 (BD Biosciences) and compensation performed using the most recent controls. Irregularities in event count (e.g. caused by momentary flow cell blockages), doublets and debris were excluded from analysis. FMO controls were considered to set gates on fluorescent markers objectively (Figure 2.5). Percentages of SSC^{hi} CD125+ CD193+ eosinophils and SSC^{lo} CD193+ CD123+ basophils among single leukocytes were determined (Figure 2.5). Eosinophil activation markers were investigated by MFI (CD125, CD193, CD69, CD123) or percentage of expression (CD16, CX3CR1); basophil activation markers by MFI (CD193, CX3CR1, CD69, CD16).

2.5.2 MDSC flow cytometry panel

For quantification of MDSC subset numbers and activation states, freshly isolated PBMC were incubated with a mixture of antibody-bound fluorochromes (Table S5). Percentage of SSC^{hi} CD66b+ CD11b+ CD14− PMN-MDSC, CD14+ HLA-DR− CD11b+ CD33+ M-MDSC and expression levels of the activation marker PD-L1 were analyzed by flow cytometry.

Assay development

The protocol was developed based on two previously established flow cytometry panels for phenotyping of PMN-MDSC (CD66b FITC) and M-MDSC (CD14 FITC, CD11b PE, HLA-DR PerCP, CD33 APC) on a BD FACSCalibur flow cytometer.[316] As the Guava easyCyte 8HT can record up to six fluorescence parameters, the two original panels were combined into one—replacing CD14 FITC (BD Biosciences) with CD14 APC-Cy7—and PD-L1 PE-Cy7 was added to assess activation status of both MDSC populations. The number of PBMC per tube was reduced from 5 x 10^5 to 2.5 x 10^5 to save more cells for later experiments. All antibodies were titrated as described (Figure S1) for optimal separation between stained and unstained cell populations.

Protocol

After PBMC isolation (Section 2.4.3), 2.5 x 10^5 cells in complete medium (RPMI, 10% FBS, 1% Pen-Strep-Glu, 1% Sodium-Pyruvate) were transferred to each of two 5 ml Fal-

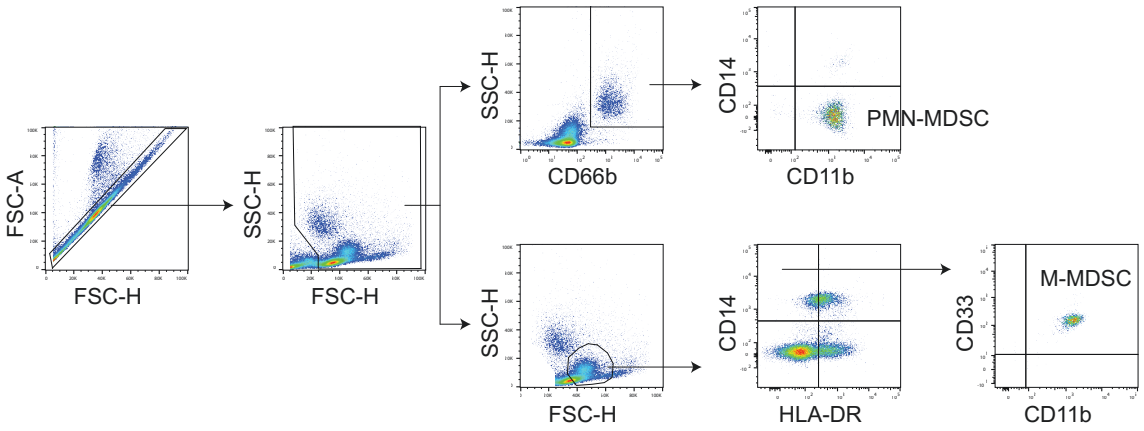

Figure 2.6: Gating strategy for PMN-MDSC and M-MDSC
Single PBMC were gated and PMN-MDSC defined as SSC^{hi} CD66b+ CD11b+ CD14−. M-MDSC were defined as SSC^{lo} HLA-DR− CD14+ CD11b+ CD33+. PD-L1 expression was assessed by MFI in both populations. Adapted from Burger et al.[235]

con tubes. 3 ml PBS was added before centrifugation (900 rpm, 10 min). The supernatant was discarded and the pellet resuspended by vortexing. 4 μl of rabbit serum dilution (Capricorn Scientific, Ebsdorfergrund, Germany; diluted 1:20 in PBS) followed by 12 μl of freshly prepared MDSC master mix (Table S5) was added to one tube, whilst the other one served as an unstained control. Both tubes were vortexed and incubated for 20 min at room temperature in the dark. 3 ml pre-cooled PBS was added and the tubes centrifuged (900 rpm, 10 min, 4°C). After discarding the supernatant, tubes were vortexed and kept on ice in the dark until acquisition as described in 2.5.1.

Compensation controls were performed weekly as described in Section 2.5.1 using MDSC panel antibodies. Because of the bead's specificity against mouse antibodies, CD11b APC (rat IgG2b) had to be replaced by CD25 APC (mouse IgG1, BD Biosciences) for compensation. FMO controls were performed once for every antibody in the panel: 2.5 x 10^5 PBMC from a healthy volunteer were incubated with five of the six antibodies used in the panel (i.e. the FITC FMO control was stained with all antibodies in the master mix except CD66b FITC). The controls were then processed and acquired according to the regular protocol.

Data analysis

FCS files were imported into FlowJo and compensation performed using the most recent controls. Irregularities in event count (e.g. caused by momentary flow cell blockages), doublets and debris were excluded from analysis. FMO controls were considered to set gates on fluorescent markers objectively. Percentages of SSC^{hi} CD66b+ CD11b+ CD14− PMN-MDSC and SSC^{lo} CD14+ HLA-DR− CD11b+ CD33+ M-MDSC among single

PBMC were determined (Figure 2.6). Expression of PD-L1 was assessed by MFI in both populations.

2.5.3 T cell proliferation-suppression assay for PMN-MDSC

Immunosuppressive capacity of PMN-MDSC can be demonstrated in a T cell proliferation-suppression assay: PMN-MDSC from a subset of study participants were isolated by magnet-activated cell sorting (MACS), targeting the PMN-MDSC marker CD66b. PBMC from a healthy volunteer were stained with carboxyfluorescein succinimidyl ester (CFSE), stimulated with interleukin-2 (IL-2) and CD3 and cultured with different concentrations of isolated PMN-MDSC. Dose-dependent CD4+ and CD8+ T cell proliferation was assessed by flow cytometry.

Assay development

The assay was largely based on a protocol established at the Institute of Tropical Medicine at the University of Tübingen.[316] Since different MACS columns were used at the CERMEL laboratory, PMN-MDSC isolation had to be refined for acceptable PMN-MDSC purity and viability: PBMC from a healthy volunteer were stained with CD66b FITC and anti-FITC MACS beads; PMN-MDSC were isolated using either a single or two consecutive isolation steps with MS columns (detailed protocol below). Isolated PMN-MDSC were investigated for purity and viability (Figure S3). Both techniques showed cell viability over 95% after MACS. Two-step isolation achieved PMN-MDSC purity of at least 90% and was applied in all further experiments.

Protocol

Healthy donor and participant PBMC were isolated as described in Section 2.4.3 with some modifications: no plasma was isolated from healthy donor blood, PBS was used for density gradient centrifugation and washing and autologous medium (RPMI 1640 without phenol red, 10% PBS/plasma supernatant from healthy donor density gradient centrifugation) for PBMC resuspension after washing.

Healthy donor PBMC were stained with CFSE, using the Vybrant CFDA SE Cell Tracer Kit (Thermo Fisher Scientific, Waltham, USA): Up to 2 x 10^7 cells were transferred to a 15 ml Falcon tube, centrifuged (1,300 rpm, 10 min) and the pellet washed in PBS (5 ml, 1,300 rpm, 10 min). The supernatant was discarded and the pellet resuspended in 2 ml CFSE dilution (0.89 μM in PBS). 4 ml FBS and 6 ml RPMI 1640 without phenol red was added after 10 min and 12 min of incubation (37°C, 5% CO_2), respectively. After centrifugation (1,300 rpm, 5 min) and discarding the supernatant, cells were

resuspended in 1 ml autologous medium, counted (Section 2.4.3) and further diluted to 5 x 10^5 PBMC/ml.

Participant PMN-MDSC were isolated by MACS: After removing 5 x 10^5 cells for the MDSC panel, remaining PBMC were transferred to a 15 ml Falcon tube, which was filled up with PBS and centrifuged (900 rpm, 10 min). The supernatant was discarded and the pellet resuspended in 100 μl PBS. 20 μl CD66b FITC was added and incubated for 20 min in the dark. 1.5 ml MACS buffer (PBS, 2% FBS, 1 mM EDTA) was added for washing (900 rpm, 10 min). The supernatant was discarded and the pellet resuspended in 90 μl MACS buffer and 10 μl anti-FITC microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min on ice in the dark. After washing (1.5 ml MACS buffer, 900 rpm, 10 min, 4°C), the supernatant was discarded and the pellet resuspended in 500 μl MACS buffer. Cells were transferred onto a pre-rinsed MS MACS column in an OctoMACS separator (both Miltenyi Biotec) and washed with 3 x 500 μl MACS buffer. The column was removed from the separator and cells pressed into a second column, using 1 ml MACS buffer and firmly applying the plunger. The procedure was repeated and cells collected in a 15 ml Falcon tube. After cell counting (Section 2.4.3) and centrifugation (900 rpm, 10 min, 4°C) the supernatant was discarded and the pellet resuspended in autologous medium to a final concentration of 5 x 10^5 cells/ml. To assess PMN-MDSC purity, up to 2.5 x 10^5 cells were transferred to a 5 ml Falcon tube. After washing (3 ml PBS, 900 rpm, 10 min) and discarding the supernatant, the resuspended pellet was transferred to a 1.5 ml Eppendorf tube prefilled with 200 μl PBS and acquired on the Guava easyCyte 8HT flow cytometer using MDSC panel settings.

Participant leukocytes were used as controls for PMN-MDSC: 200 μl of full blood was incubated with 5 ml FACS lysing solution for 10 min. After centrifugation (2,000 rpm, 5 min) and discarding the supernatant cells were washed twice in 5 ml RPMI 1640 without phenol red, 1% Pen-Strep-Glu (2 000 rpm, 5 min). The pellet was resuspended in 0.5 ml autologous medium and diluted to 5 x 10^5 cells/ml after counting.

Duplicate unstimulated controls were removed to a 96-well round-bottom plate before incubating the remaining PBMC with anti-human CD3 (BD Biosciences; 1.5 μg/ml) and IL-2 (R&D Systems, Minneapolis, USA; 150 U/ml) for 10 min. 6 x 10^5 PBMC were transferred to each well and PMN-MDSC or leukocyte controls added at concentrations of 1:1, 1:2, 1:4, 1:8, 1:16 and 0 (positive controls). All wells were filled up to 180 μl autologous medium and cells cultured for 96 hours at 37°C, 5% CO_2.

Cells were harvested to 5 ml Falcon tubes and washed in 3 ml PBS (1,500 rpm, 3 min). The supernatant was discarded and the resuspended pellet incubated with 1 μl CD4 PE and 1 μl CD8a APC (both BioLegend, San Diego, USA) for 15 min in the dark. After washing (3 ml PBS, 1,500 rpm, 3 min) the tubes were transferred on ice. 1 μl propidium

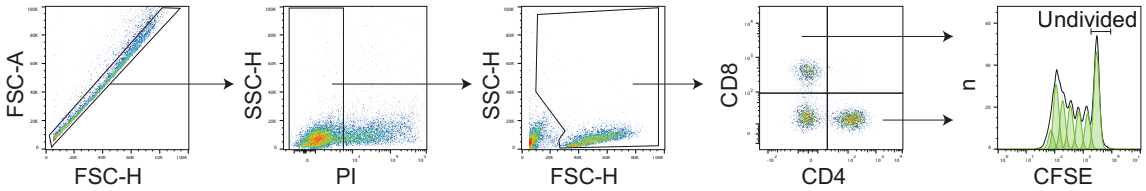

Figure 2.7: Proliferation assessment of CD4+ and CD8+ T cells by CFSE staining
Proliferation of CD4+ and CD8+ T cells was evaluated by flow cytometry after a 96-hour proliferation-suppression assay: Single live CD4+ and CD8+ lymphocytes were gated and expression of CFSE investigated by FlowJo Proliferation Modelling. Proliferation index was calculated as the total number of cell divisions divided by the number of cells that went into division. Adapted from Burger et al.[235]

iodide (Thermo Fisher Scientific; 1.0 mg/ml) was added to the resuspended pellet for 3 min and cells transferred to a 1.5 ml Eppendorf tube prefilled with 100 μl PBS before acquisition on the Guava easyCyte 8 HT flow cytometer.

Compensation controls were performed once using 10^5 healthy donor PBMC. CD4 PE and CD8a APC controls were treated as above. For propidium iodide, cells were heat-shocked at 60°C for 10 min before staining to assure a sufficient dead cell (positive) population. For CFSE, 5 x 10^4 of each unstained and CFSE-stained PBMC were combined in the same tube.

Data analysis

FCS files were imported to FlowJo for data analysis. Purity of PMN-MDSC after MACS isolation was assessed as percentage of single cells expressing CD66b (Figure S3). Live single cells were gated and CD4+ and CD8+ lymphocytes investigated for CFSE expression (Figure 2.7). FlowJo Proliferation Modelling was used to calculate proliferation index, defined as total number of cell divisions divided by the number of cells that went into division.[349]

2.5.4 Cytometric bead array for circulating cytokine detection

A cytometric bead array was performed to measure circulating cytokine levels associated with the innate cell populations under investigation in plasma samples by flow cytometry.

Assay development

The protocol was set up according to the manufacturer's recommendations. Instrument setup beads and the top standards (see below) were acquired to determine photomultiplier (PMT) voltages and set compensation. The minimal recommended dilution for plasma samples (1:4) was selected for maximal assay sensitivity.

Protocol

Cytometric bead array (CBA) Human Soluble Protein Master Buffer Kit and human IL-1b, IL-4, IL-5, IL-6, IL-10, IFN-γ, TNF-α and GM-CSF flex sets (BD Biosciences, San Jose, USA) were used according to the manufacturer's specifications: Capture beads were mixed, washed in 0.5 ml wash buffer (200 G, 5 min), resuspended in capture bead diluent for serum/plasma to a final concentration of 1:50 and incubated for 15 min prior to use. PE detection reagents were mixed and diluted to a final concentration of 1:50 with detection reagent diluent. Lyophilized standard spheres from each flex set were reconstituted for 15 min with 4 ml assay diluent in a 15 ml Falcon tube. A serial dilution was performed to prepare 500 μl top, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024 standards. Assay diluent served as a negative control. Plasma was thawed at room temperature and diluted 1:4 with assay diluent. 50 μl of standards and diluted plasma samples was added to 1.5 ml Eppendorf tubes with 50 μl of mixed capture beads and incubated for 1 h. 50 μl of mixed PE detection reagent was added and incubated for another 2 h in the dark. 1 ml of wash buffer was added before centrifugation (200 G, 5 min). The supernatant was discarded and the pellet resuspended in 300 μl wash buffer before acquisition on the Guava easyCyte 8HT flow cytometer.

Data analysis

FCS files were imported into FlowJo for data analysis. Compensation was performed with A1, F1 and F9 instrument setup beads. Single beads were gated for each cytokine according to their fluorescence signature and MFI of the reporter antibody calculated.

2.6 Statistical analyses

All data were collected with FlowJo version 10.6.1 (BD Biosciences, San Jose, USA), Excel version 1908 (Microsoft, Redmond, USA) and REDCap research electronic data capture system version 8.3.1 (Vanderbilt University, Nashville USA).[350,351] Data cleaning, analysis and visualization were performed in R version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria) within the tidyverse package ecosystem.[352,353]

Categorical data were summarized by frequency and numeric data by median and interquartile range (IQR). Microfilaraemia of participants in the treatment study was normalized to the mean of screening and baseline microfilaraemia due to high between-subject variation and summarized by median per treatment group. Proliferation indices in the T cell proliferation-suppression assay were normalized to the positive control performed on the respective study day. Cytokine concentrations in the cytometric bead array were derived from standard curves by a four-parameter variable slope model of the

log-transformed standard MFI and concentration values, where the bottom variable was constrained to the log-transformed MFI of the negative controls. Measurements below the lower limit of detection (LLOD) were set to half the LLOD.

Multivariable logistic regression analysis and Wald tests were performed to assess factors associated with *Loa loa* microfilaraemia in the screening population. Friedman tests were used to assess between-subject differences in the cross-sectional study and within-subject differences across multiple time points in the treatment study. Nemenyi's post hoc test for multiple comparisons was applied after significant Friedman tests. Linear regression analysis and Spearman rank correlation were used to investigate the relationship between immunological parameters and microfilaraemia. Two-sided p-values < 0.05 were considered statistically significant.

3 Results

3.1 Study population

3.1.1 Screening

A total of 605 inhabitants of the Gabonese departments Estuaire, Moyen-Ogooué and Ngounié participated in screening for this study throughout dry and rainy seasons between May 2018 and December 2020 (Figure 3.1). The screened population was relatively old (median age, interquartile range: 37, 21–58 years), with most participants being older than 40 years (Table 3.1). Slightly more women than men were screened (56% female). The prevalence of history of eye worm (positive RAPLOA) was high (38%). The prevalence of *Loa loa* microfilaraemia was 22% but the median microfilaraemia was quite low: 63 mf/ml (IQR 6–909). Forty-seven % of individuals either reported history of eye worm or had microscopically confirmed *Loa loa*-microfilaraemia. Almost a third (29%) of the study population had a positive malaria rapid diagnostic test (RDT). Participants with history of eye worm (50, 32–63 years) or *Loa loa* microfilaraemia (55, 35–69 years) were significantly older than the average screening population, while malaria-diagnosed participants were significantly younger (27, 12–54 years; Figure S4). Male sex (Odds ratio 2.30, 95% confidence interval 1.46–3.68), age > 40 (4.27, 1.89–11.02) and history of eye worm (2.76, 1.74–4.44), but not malaria infection (1.22, 0.74–2.01) were associated with higher risk for *Loa loa* microfilaraemia in multivariable logistic regression analysis (Figure 3.2). In microfilaraemic participants we found a trend to higher microfilaraemia for subjects with history of eye worm (105, 7–1,810 vs 31, 5–245 mf/ml; Figure S5). There was no association of microfilarial density with sex or age (Figure S5).

3.1.2 Cross-sectional case-control study

Forty-two individuals could be included into the cross-sectional study (Figure 3.1). Matched inclusion was limited due to malaria infection and the scarcity of participants corresponding to the uninfected control group definition. Moreover, eligible *Loa loa*-uninfected participants were relatively young (31, 16–36 years) compared to eligible microfilaraemic individuals (54, 32–76 years), which further impeded the selection of age-matched participants. We included 33 female and 9 male participants (Table 3.2). The median age was 25 years and did not differ significantly between microfilaraemic (MF+), amicrofilaraemic (MF-) and *Loa loa*-uninfected (LL-) participants (p = 0.61). Median *Loa loa* microfilaraemia in MF+ was low: 26 mf/ml (3–150). One MF- and two MF+ participants were co-infected with *Mansonella perstans*. Data from the eosinophil/basophil and MDSC panel were available for 39 and 42 participants, respectively.

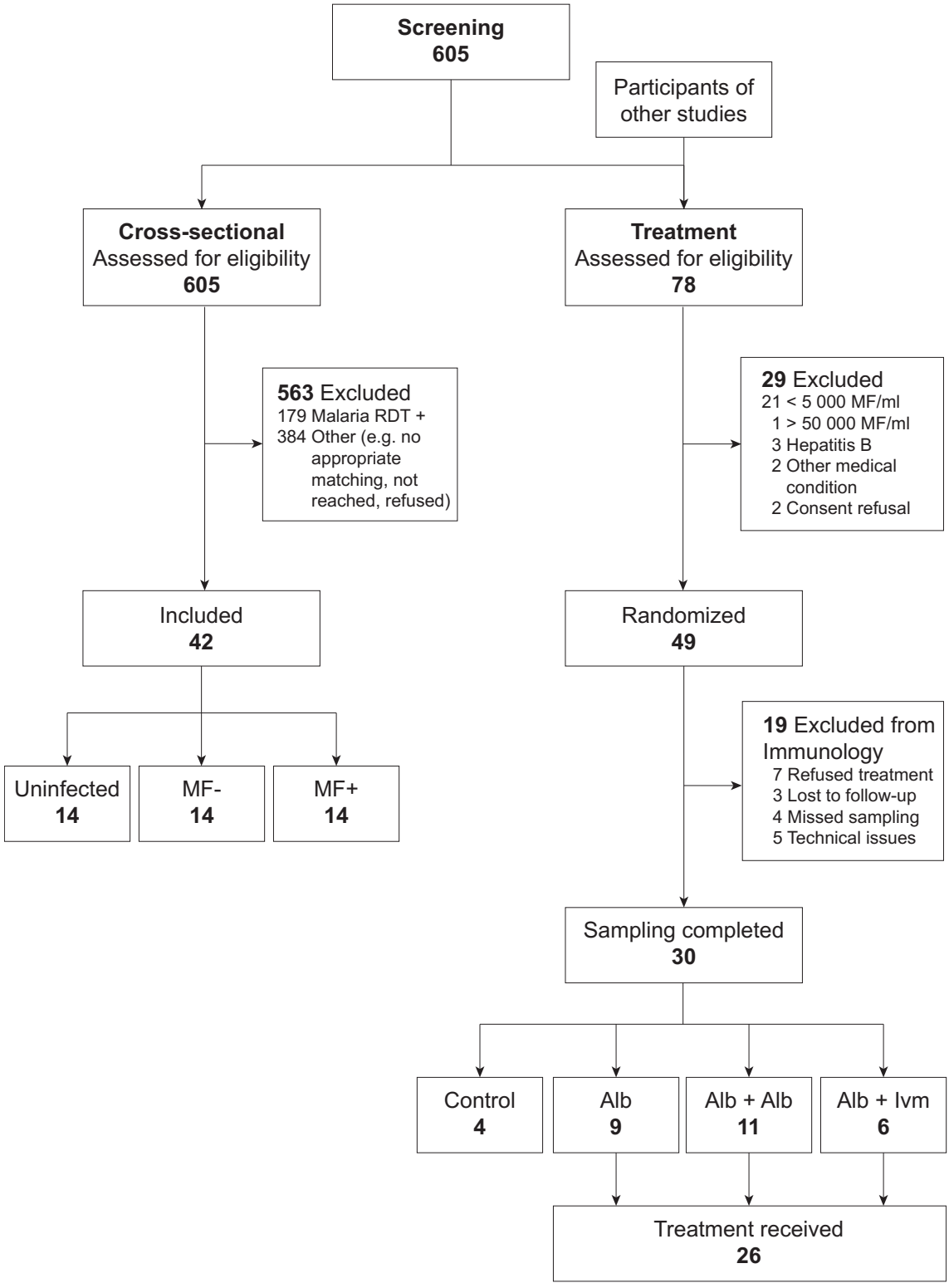

Figure 3.1: Study population
MF-: amicrofilaraemic, MF+: microfilaraemic, Alb: albendazole, Ivm: ivermectin.

Table 3.1: Characteristics of the screening population

Characteristic		Total number	Percentage (%)
Sex	Female	341	56.4
	Male	264	43.6
Age, yr.	2–14	106	17.5
	15–40	208	34.4
	$>$ 40	291	48.1
Clinical examination	History of eye worm	231	38.2
	Loa loa microfilaraemia	135	22.3
	Insufficient microscopy*	62	10.2
	Positive malaria RDT	179	29.6
	No malaria RDT	38	6.3

* e.g. additional leukocyte concentration was not performed after negative TBS microscopy in some participants already ineligible by a positive malaria RDT.

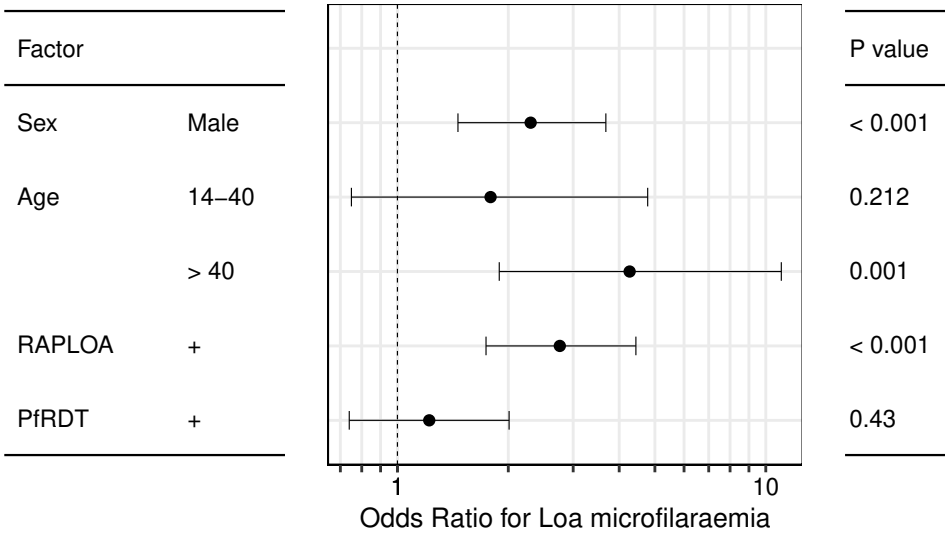

Figure 3.2: Risk factors for *Loa loa* microfilaraemia in the screening population
Odds Ratios were derived from a multivariable logistic regression model including participant characteristics and diagnostics performed at screening.

3.1.3 Randomized controlled trial

In the randomized controlled trial, 78 individuals were assessed for eligibility (Figure 3.1). Major reasons for exclusion were low microfilaraemia ($<$ 5,000 mf/ml), hepatitis, other medical conditions (e.g. HIV infection) and microfilaraemia above the

Table 3.2: Characteristics of the cross-sectional population[235]

Characteristic	Uninfected (n = 14)	Amicrofilaraemic (n = 14)	Microfilaraemic (n = 14)
Female sex, no. (%)	11 (79)	11 (79)	11 (79)
Median age, yr. (IQR)	28 (16–42)	24 (16–44)	24 (15–47)
Median *Loa loa*, mf/ml (IQR)			26 (3–150)

Table 3.3: Characteristics of the treatment population at baseline

	Control (n = 4)	3 w Alb (n = 9)	5 w Alb (n = 11)	3 w Alb + Ivm (n = 6)
Female sex, no. (%)	0 (0)	5 (56)	5 (45)	3 (50)
Median age, yr (IQR)	52 (47–56)	66 (61–76)	57 (45–67)	65 (61–71)
Median *Loa loa*, mf/ml (IQR)	12,800 (7,500–18,200)	10,250 (7,400–12,400)	13,600 (11,200–21,300)	8,550 (6,080–9,680)
History of eye worm, no. (%)	4 (100)	6 (67)	10 (91)	5 (83)
History of Calabar swelling, no. (%)	2 (50)	2 (22)	6 (55)	4 (67)
Pruritus, no. (%)	3 (75)	7 (78)	9 (82)	3 (50)
Headache, no. (%)	3 (75)	7 (78)	9 (82)	3 (50)
Myalgia, no. (%)	1 (25)	4 (44)	8 (73)	3 (50)
Arthralgia, no. (%)	2 (50)	4 (44)	9 (82)	2 (33)

security threshold of 50,000 mf/ml. Among the 49 randomized participants, 30 (61%) completed immunology (data available for day 0, 14 and 168) due to consent withdrawal, loss to follow-up, incomplete sampling or technical issues in the laboratory in the remainder. Due to limited data from the subgroups that received a second round of albendazole or ivermectin, we analyzed the pooled data from all three treatment groups (n = 26), excluding the control group (n = 4). The study interventions, including drug exposure, were identical for all these participants until day 21, encompassing the immunological assessment on day 14. Complete data from the eosinophil/basophil or MDSC panel were available for 22 participants, respectively.

Table 3.3 shows the baseline characteristics of individuals in each treatment arm. Participants in the treated population (n = 26) had a balanced sex distribution across treatment groups and a median age of 62 (48–72) years. Prevalence of clinical *Loa loa* signs was high, with history of eye worm (81%), pruritus (73%) and headache (73%) being the most

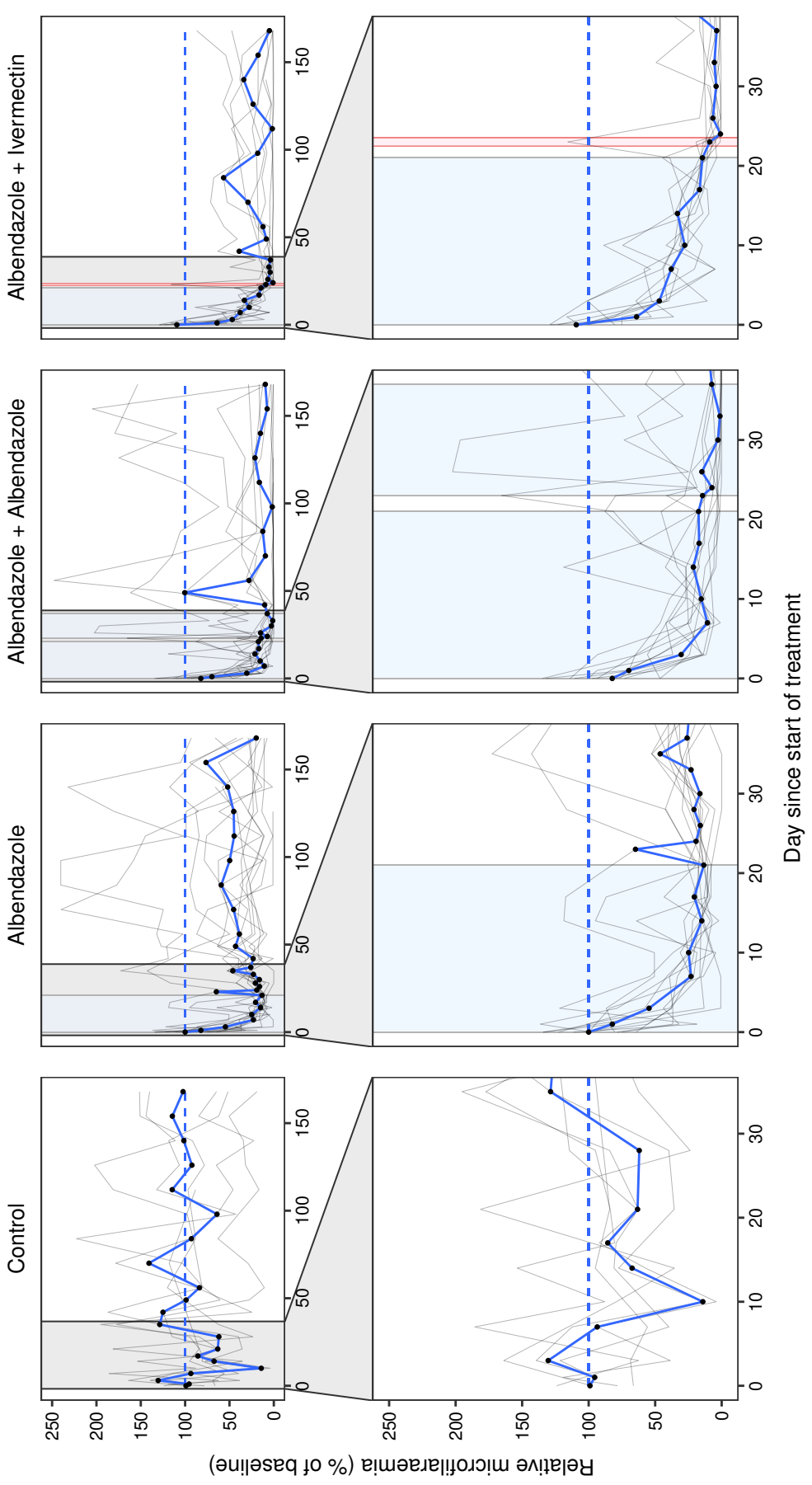

Figure 3.3: Relative microfilaraemia by treatment group
Microfilaraemia in each treatment group until end of follow-up and during treatment with albendazole (blue) and ivermectin (red). Relative microfilaraemia was standardized to the mean of screening and baseline microfilaraemia (blue dashed lines, set to 100%) for 30 indiviual participants (grey lines). Black dots represent the median relative microfilaraemia.

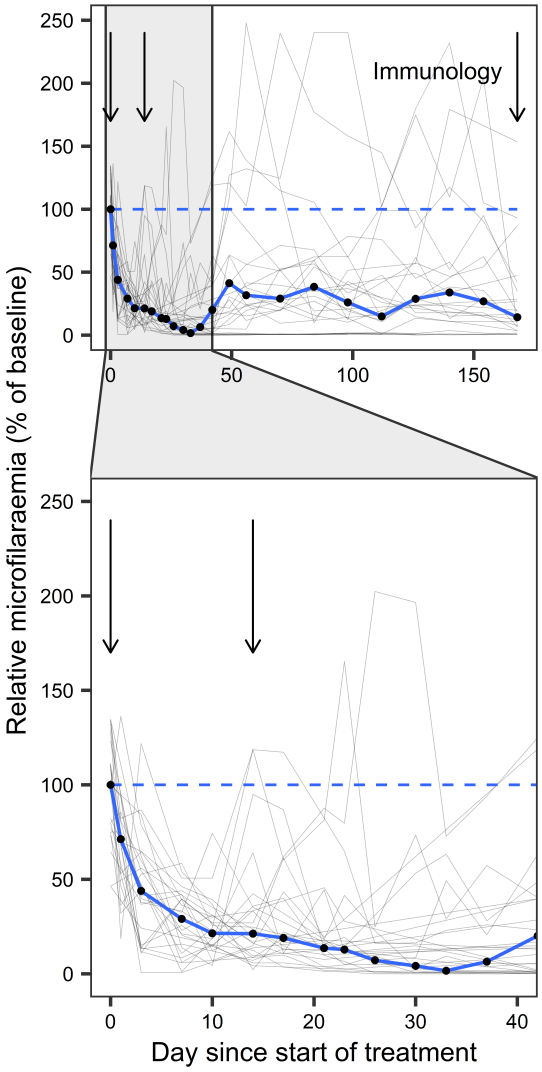

Figure 3.4: Relative microfilaraemia in 26 treated individuals
Microfilaraemia in 26 treated individuals until end of follow-up. Relative microfilaraemia was standardized to the mean of screening and baseline microfilaraemia (blue dashed lines, set to 100%) for individual participants (grey lines). Black dots represent the median relative microfilaraemia and arrows sampling for immunology. Adapted from Burger et al.[235]

common symptoms. Baseline stool microscopy could be performed in 18/26 (69%) and was positive in 7/18 (39%) treated individuals (Table S6). Infection with *M. perstans* was present in 8/26 (31%) participants.

Figure 3.3 displays relative *Loa loa* microfilaraemia (percentage of mean screening and baseline microfilaraemia) of each group over time in the treatment study. In untreated participants, median relative microfilaraemia varied widely (± 50%) over the course of the study. In the 26 participants treated with albendazole, it dropped to 24% of baseline (median absolute microfilaraemia: 2,200 mf/ml) on day 14 and 16% (1,400 mf/ml) on

Table 3.4: Number of adverse event episodes until day 40

Clinical symptom	Control (n = 4)	3 w Alb (n = 9)	5 w Alb (n = 11)	3 w Alb + Ivm (n = 6)	Total (n = 30)
Headache	0	2	7	2	11
Diarrhea	0	1	3	0	4
Fever	0	2	1	1	4
Vertigo	0	1	3	0	4
Malaria	0	0	1	2	3
Back pain	0	1	0	1	2
Fatigue	2	0	0	0	2
Nausea	0	0	0	2	2
Other	0	2	1	1	4
Total	2	9	16	9	36

day 21. After treatment with ivermectin, median relative microfilaraemia decreased from 9% (800 mf/ml) on day 23 to 1% (100 mf/ml) on day 24. Reduction of microfilaraemia did not extend beyond the treatment period: nadir of median microfilaraemia was reached on day 21 of a three-week course albendazole (13%, 1,400 mf/ml, group two), on day 33 of a three week-course followed immediately by another two-week course albendazole (1%, 175 mf/ml, group three) and on day 24 of a three-week course albendazole followed by a single dose of ivermectin (1%, 100 mf/ml, group four), after which microfilaraemia gradually recrudesced. Mean relative microfilaraemia of day 156 and 168 at the end of follow-up reached 48% (4,800 mf/ml) in group two, 9% (1,000 mf/ml) in group three and 11% (800 mf/ml) in group four.

Over the course of treatment (until day 40), 36 adverse events were recorded. Headache, diarrhea, fever and vertigo were the most frequent adverse events (Table 3.4). Timing of adverse events per group is presented in Figure S6. Adverse event incidence peaked around day 14–21 in treated individuals (Figure S7). There were no significant differences in adverse event incidence between untreated and treated individuals (Table S7).

3.2 Eosinophils

3.2.1 Eosinophil numbers

Eosinophilia—percentage of CD125+ CD193+ eosinophils among leukocytes—was higher in *Loa loa*-infected than uninfected (LL-: 3.0%, 1.8–5.0) participants, but did not differ between microfilaraemic (MF+: 13.1%, 9.0–24.5) and amicrofilaraemic (MF-: 12.3%, 10.2–19.2) individuals in the cross-sectional study (Figure 3.5). In the treatment

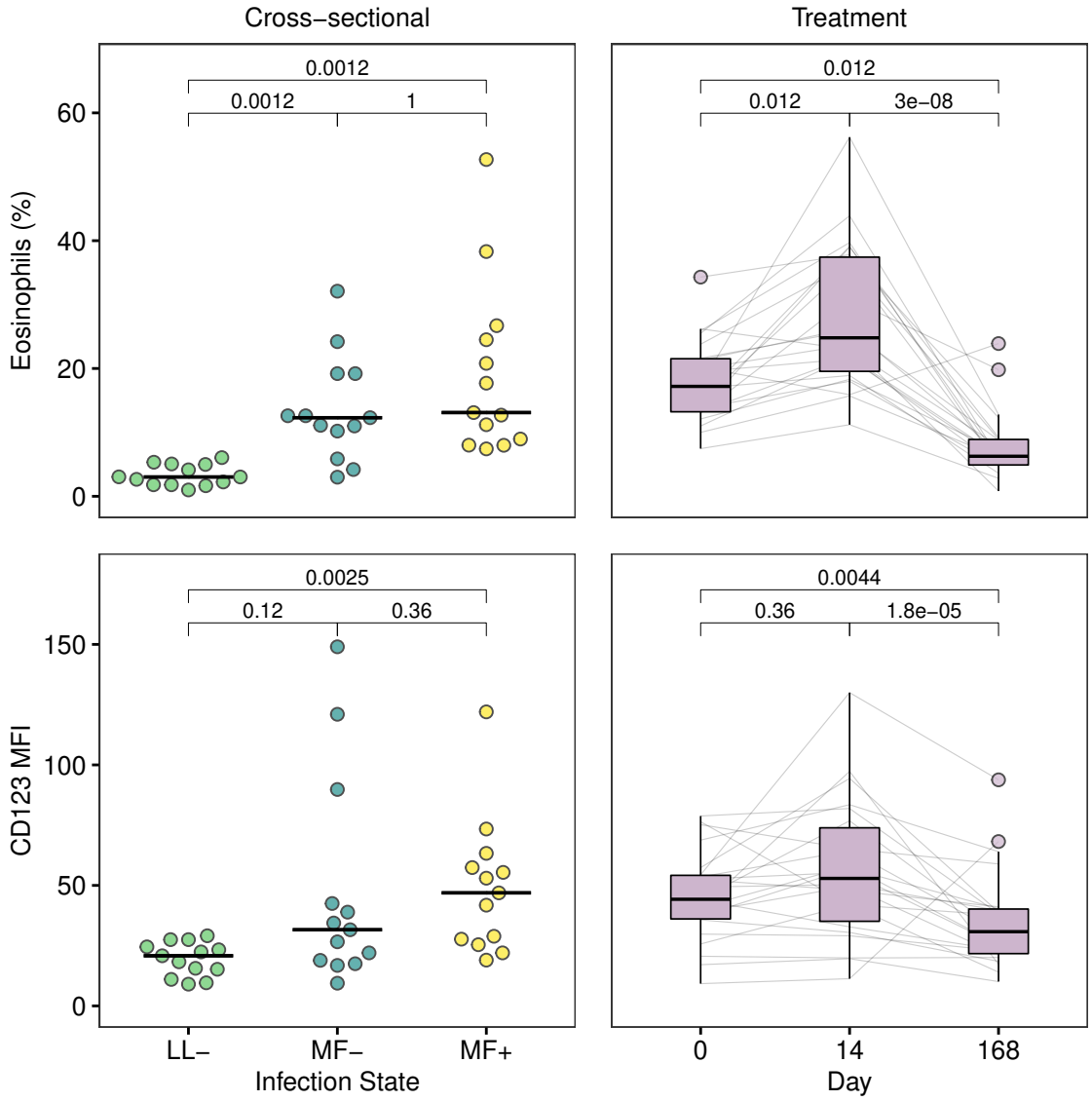

Figure 3.5: Eosinophil numbers and CD123 expression
Percentage of CD125+ CD193+ eosinophils among single leukocytes (top) and CD123 median fluorescence intensity (MFI, bottom) in 39 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left) and 22 microfilaraemic individuals upon treatment (right). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

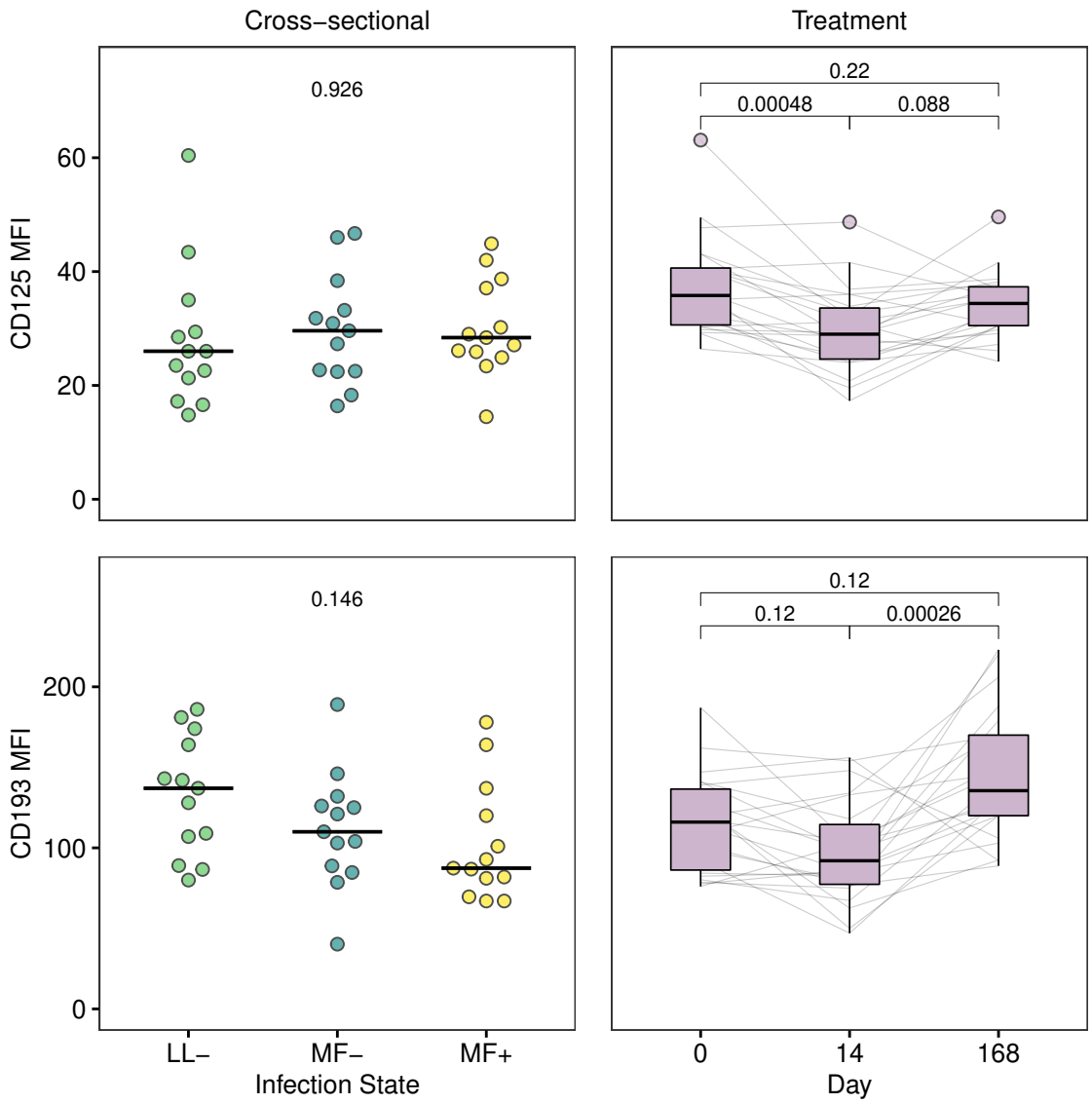

Figure 3.6: CD125 and CD193 expression of eosinophils
CD125 (top) and CD193 (bottom) median fluorescence intensity (MFI) of CD125+ CD193+ eosinophils in 39 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left) and 22 microfilaraemic individuals upon treatment (right). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

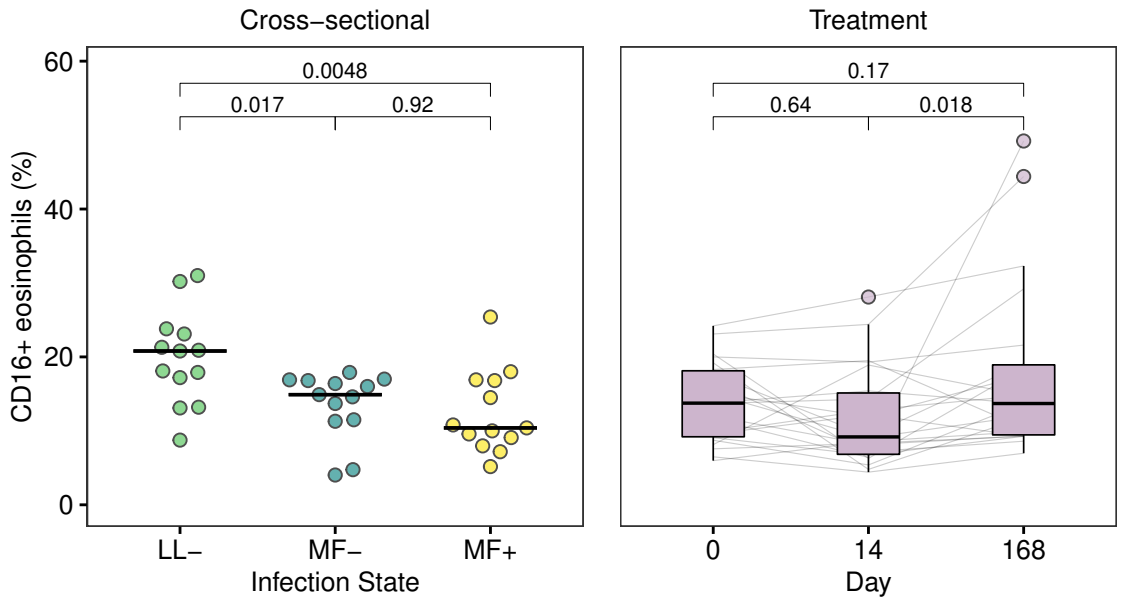

Figure 3.7: CD16 expression of eosinophils
Percentage of CD125+ CD193+ eosinophils expressing CD16 in 39 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left panel) and 22 microfilaraemic individuals upon treatment (right panel). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

study, baseline eosinophil numbers were high (17.2%, 13.2–21.5) and markedly increased during treatment with albendazole at day 14 (24.8%, 19.5–37.4), followed by a clear decrease below baseline at the end of follow-up (6.3%, 4.9–8.9; Figure 3.5).

3.2.2 Eosinophil activation markers

Eosinophil expression of the activation marker CD123 (conveyed as MFI) was higher in MF+ (46.9, 27.7–57.4) than MF- (31.6, 18.9–42.5) than LL- (20.8, 15.2–24.5) in the cross-sectional study, although only the difference between MF+ and LL- reached statistical significance (Figure 3.5). Similarly to changes observed in eosinophil numbers, CD123 expression in the treatment study trended to increase from baseline (44.2, 36.1–54.2) to day 14 (52.9, 35.1–73.9), followed by a clear decrease below baseline at day 168 (30.8, 21.7–40.2; Figure 3.5). Expression of the reverse activation marker CD125 did not differ between MF+ (28.4, 25.9–37.1), MF- (29.6, 22.5–33.2) and LL- (26.0, 21.3–29.4) in the cross-sectional study (Figure 3.6). However, in the treatment study—conversely to eosinophil numbers and CD123 expression—CD125 MFI decreased significantly between baseline (35.8, 30.6–40.6) and day 14 (29.0, 24.6–33.6), and trended to increase until day 168 (34.4, 30.5–37.3; Figure 3.6). Eosinophil expression of CD193, which is known to be downregulated in activated eosinophils, trended to be lower in MF+ (87.4,

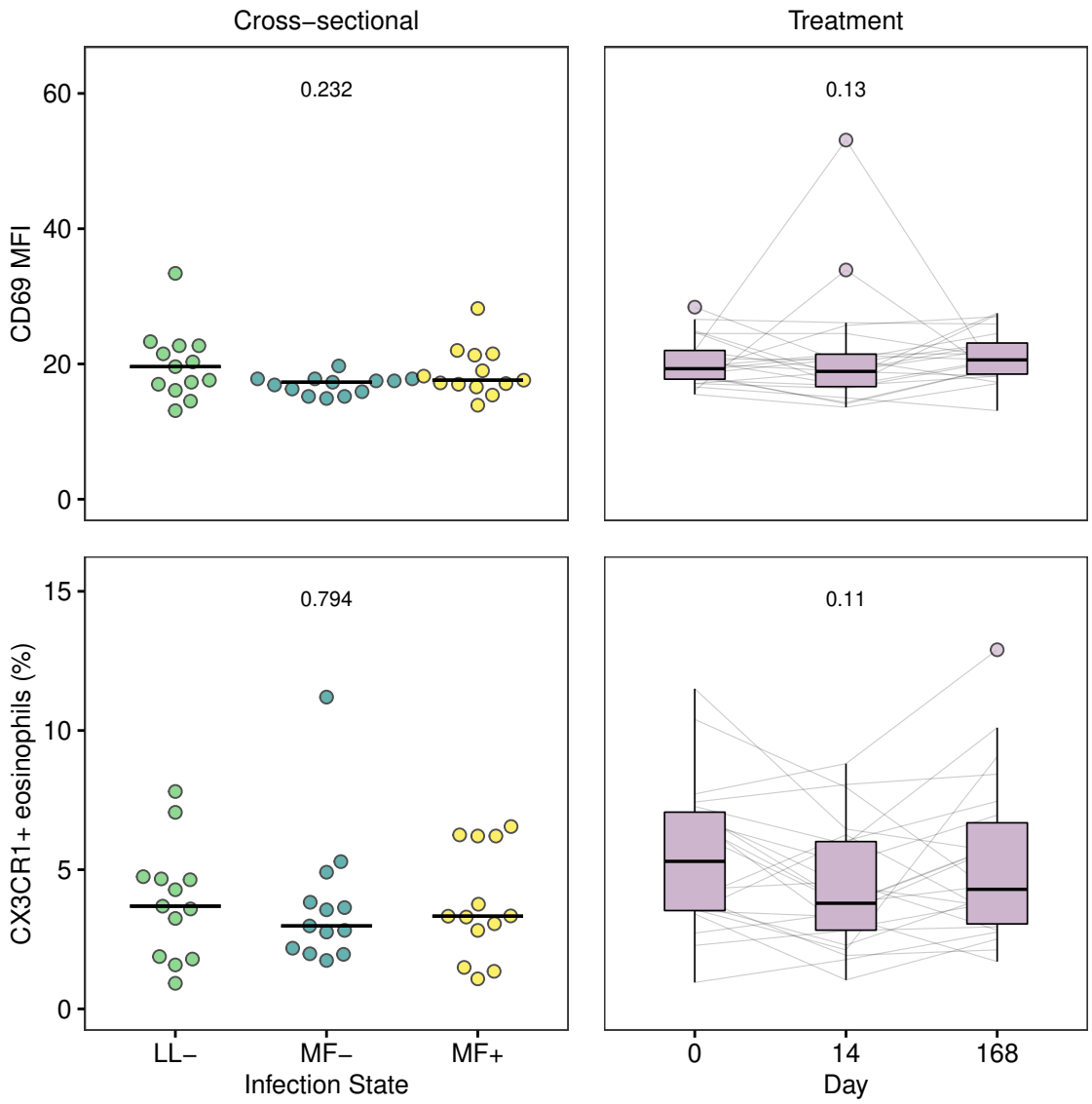

Figure 3.8: CD69 and CX3CR1 expression of eosinophils
CD69 median fluorescence intensity (MFI, top) of CD125+ CD193+ eosinophils and percentage expressing CX3CR1 (bottom) in 39 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left) and 22 microfilaraemic individuals upon treatment (right). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

81.1–120) than MF- (110, 88.8–126) than LL- (137, 107–164) participants (Figure 3.6). Upon treatment CD193 MFI trended to decrease between baseline (116, 86.2–136) and day 14 (92.0, 77.4–114.0), followed by a marked increase until the end of follow-up (136, 120–170; Figure 3.6). The percentage of eosinophils expressing CD16—which is considered a sign of activation—was *lower* in MF+ (10.4%, 9.1–16.8) and MF- (14.9%, 11.5–16.8) than LL- (20.8%, 17.2–23.1) individuals in the cross-sectional study (Figure 3.7). In the treatment study, percentage of CD16+ eosinophils slightly decreased between baseline (13.8%, 9.2–18.1) and day 14 (9.18%, 6.8–15.1) and significantly increased until day 168 (13.7%, 9.5–18.9; Figure 3.7). Eosinophil expression of the activation marker CD69 did not differ between MF+ (17.6, 17.0–21.3), MF- (17.3, 15.9–17.8) and LL- (19.6, 17.0–22.7) individuals in the cross-sectional study; nor between baseline (19.3, 17.8–22.0), day 14 (18.9, 16.7–21.4) and end of follow-up (20.6, 18.5–23.1) in the treatment study (Figure 3.8). Finally, the percentage of eosinophils expressing the activation marker CX3CR1 did not differ between MF+ (3.3%, 2.8–6.2), MF- (3.0%, 2.2–3.8) and LL- (3.7%, 1.9–4.7) participants. In the treatment study percentage of CX3CR1+ eosinophils trended to slightly decrease between baseline (5.3%, 3.5–7.1) and day 14 (3.8%, 2.8–6.0), and increase until the end of follow-up (4.3%, 3.1–6.7; Figure 3.8).

3.2.3 Eosinophil parameters and microfilaraemia

When correlating eosinophil parameters and microfilaraemia at baseline (Figure 3.9 & 3.10), some associations were observed: CD16 expression of eosinophils was inversely correlated with microfilarial density in the treatment study ($R = -0.43$, $p = 0.0031$; Figure 3.10). A similar trend was observed in MF+ individuals in the cross-sectional ($R = -0.36$, $p = 0.2$), but not in the combined microfilaraemic population ($R = -0.15$, $p = 0.38$, including MF+ individuals from both cross-sectional and treatment study at baseline). Eosinophil CD69 MFI correlated inversely with microfilaraemia in the treatment study ($R = -0.44$, $p = 0.026$), but not in the MF+ ($R = -0.2$, $p = 0.5$) or combined microfilaraemic population ($R = -0.04$, $p = 0.81$; Figure 3.10). Percentage of eosinophils expressing CX3CR1 correlated positively with microfilarial density only in the combined microfilaraemic population ($R = 0.39$, $p = 0.015$), but not in the cross-sectional MF+ ($R = 0.029$, $p = 0.92$) or treatment populations ($R = 0.14$, $p = 0.49$) separately.

When correlating eosinophil parameters at day 14 and drop in microfilaraemia between baseline and day 14 as percentages of baseline in the treatment study (Figures S8–S14), only increase in CD69 expression correlated positively with drop in microfilaraemia ($R = 0.55$, $p = 0.0089$; Figure S13).

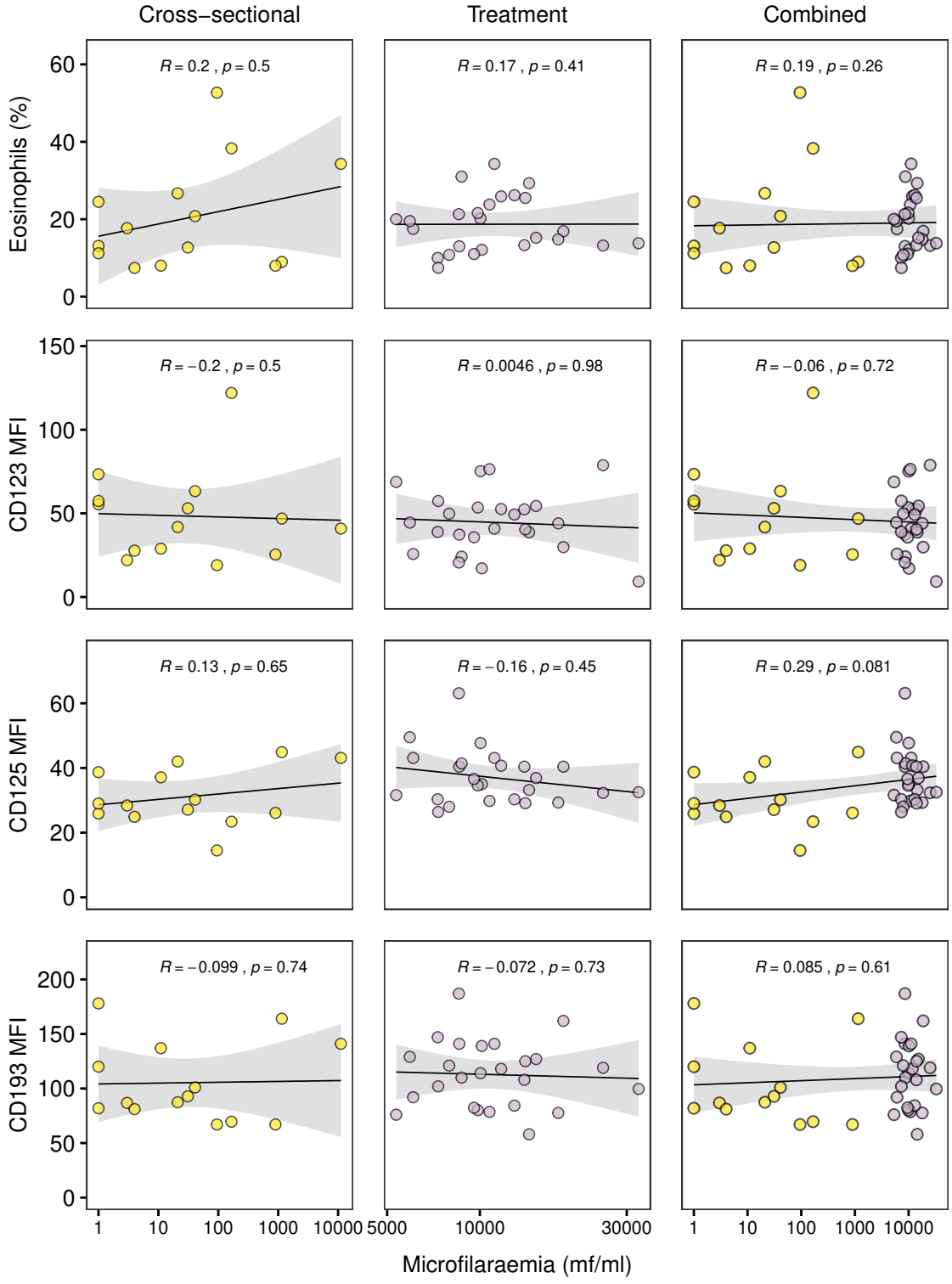

Figure 3.9: Correlation of eosinophil parameters and baseline microfilaraemia (1/2)
Correlation of percentage of CD125+ CD193+ eosinophils among single leukocytes and expression of surface activation markers as median fluorescence intensity (MFI) and baseline *Loa loa*-microfilaraemia in 14 participants in the cross-sectional (left), 25 participants in the treatment study (middle) and the combined microfilaraemic population (right). Black lines and shaded areas represent linear regression lines with 95% confidence bands. R and p values were obtained by Spearman correlation analysis. Adapted from Burger et al.[235]

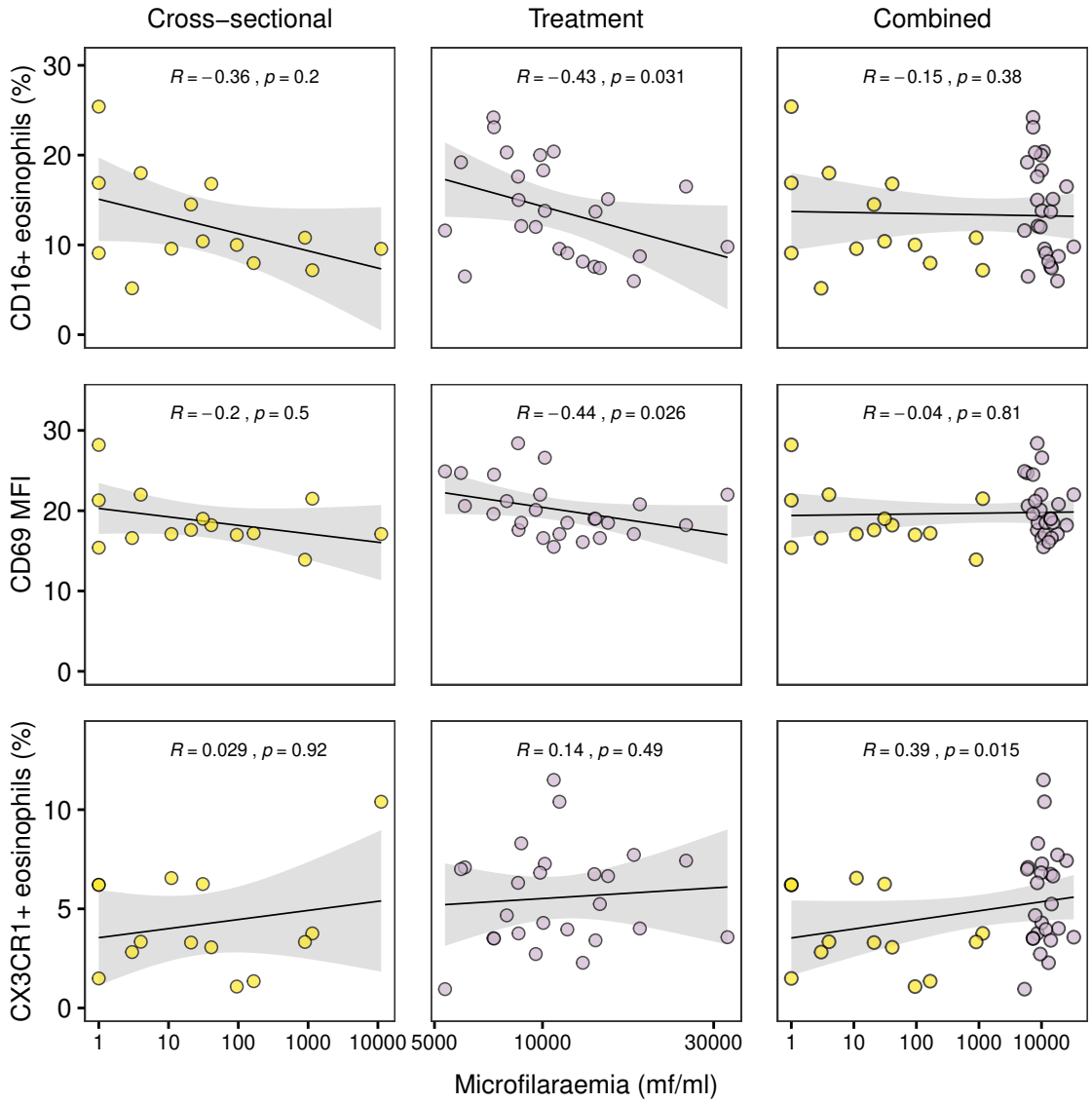

Figure 3.10: Correlation of eosinophil parameters and baseline microfilaraemia (2/2)
Correlation of eosinophil expression of surface activation markers as median fluorescence intensity (MFI) or percentage positive and baseline *Loa loa*-microfilaraemia in 14 participants in the cross-sectional (left), 25 participants in the treatment study (middle) and the combined microfilaraemic population (right). Black lines and shaded areas represent linear regression lines with 95% confidence bands. R and p values were obtained by Spearman correlation analysis. Adapted from Burger et al.[235]

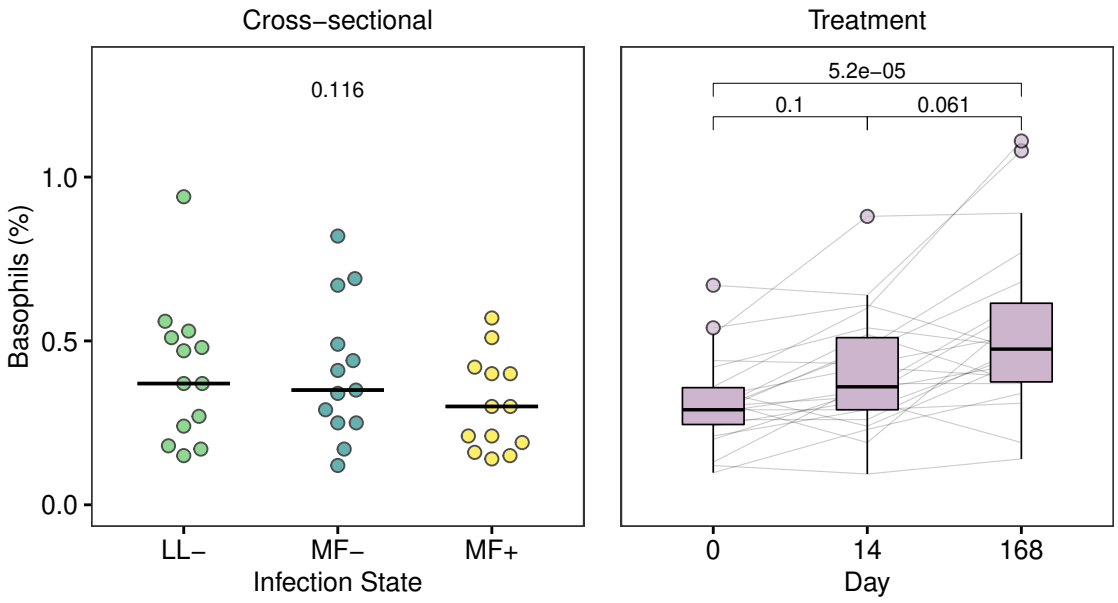

Figure 3.11: Basophil numbers
Percentage of CD123+ CD193+ basophils among single leukocytes in 39 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left panel) and 22 microfilaraemic individuals upon treatment (right panel). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

3.3 Basophils

3.3.1 Basophil numbers

Basophil numbers—percentage of CD123+ CD193+ basophils among leukocytes—did not differ between MF+ (0.30%, 0.19–0.40), MF- (0.35%, 0.25–0.49) and LL- (0.37%, 0.24–0.51) individuals in the cross-sectional study (Figure 3.11). However, in the treatment study basophil numbers trended to increase between baseline (0.29%, 0.25–0.36) and day 14 (0.36%, 0.29–0.51), followed by a marked increase until day 168 (0.48%, 0.38–0.62; Figure 3.11).

3.3.2 Basophil activation markers

CD193 MFI of basophils did not differ between MF+ (124, 111–154), MF- (132, 113–169) and LL- (157, 135–163) individuals in the cross-sectional study (Figure 3.12). However, upon treatment basophil CD193 expression—very similarly to eosinophil CD193 expression—trended to slightly decrease between baseline (144, 124–180) and day 14 (135, 126–152), followed by a marked increase above baseline until the end of follow-up (176, 148–202; Figure 3.12). Basophil expression of the activation marker CD69 did not differ between MF+ (5.9, 5.1–6.6), MF- (6.1, 5.3–7.3) and LL- (7.2, 5.7–8.5) individuals

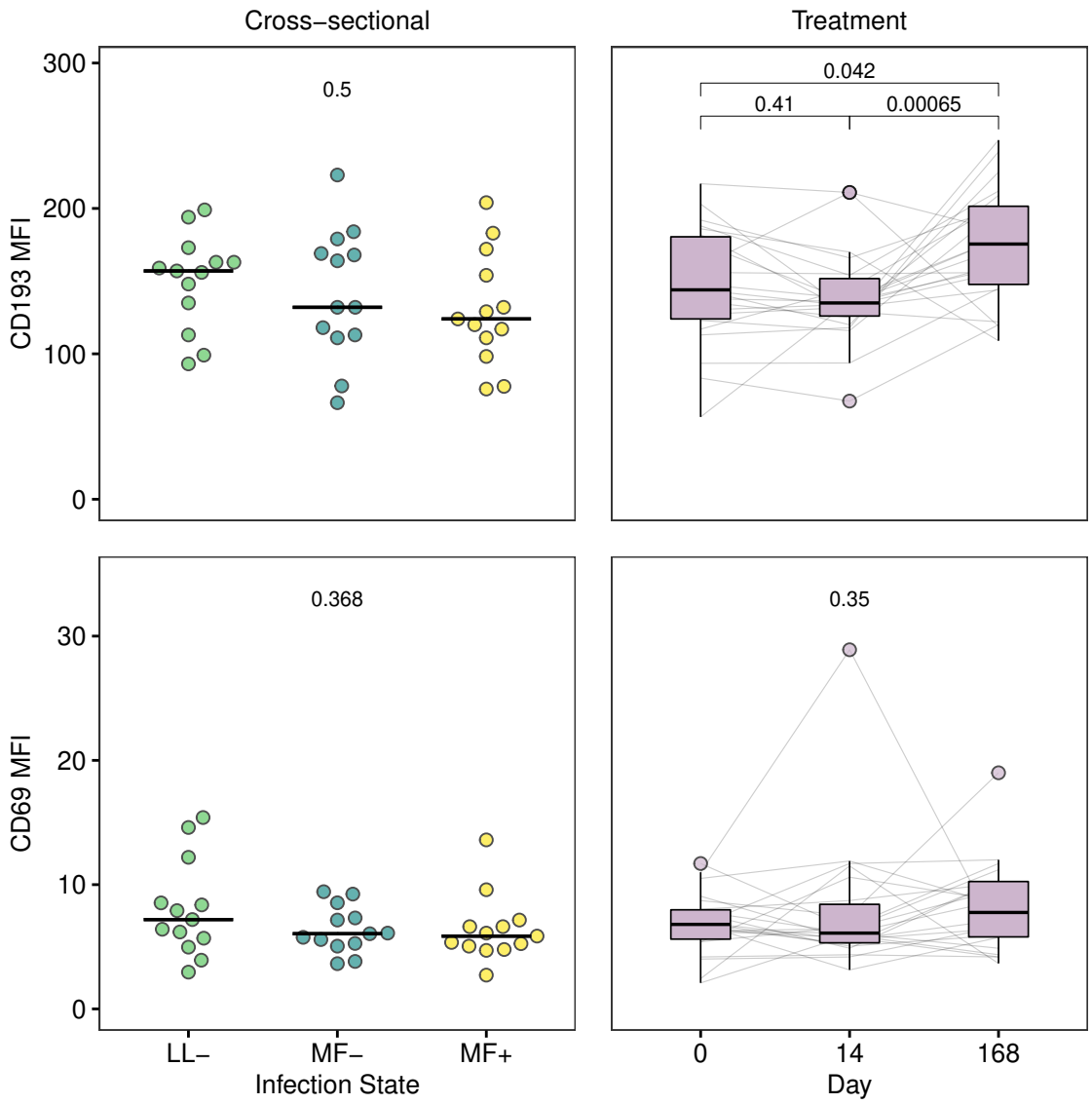

Figure 3.12: CD193 and CD69 expression of basophils
CD193 (top) and CD69 (bottom) median fluorescence intensity (MFI) of CD123+ CD193+ basophils in 39 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left) and 22 microfilaraemic individuals upon treatment (right). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

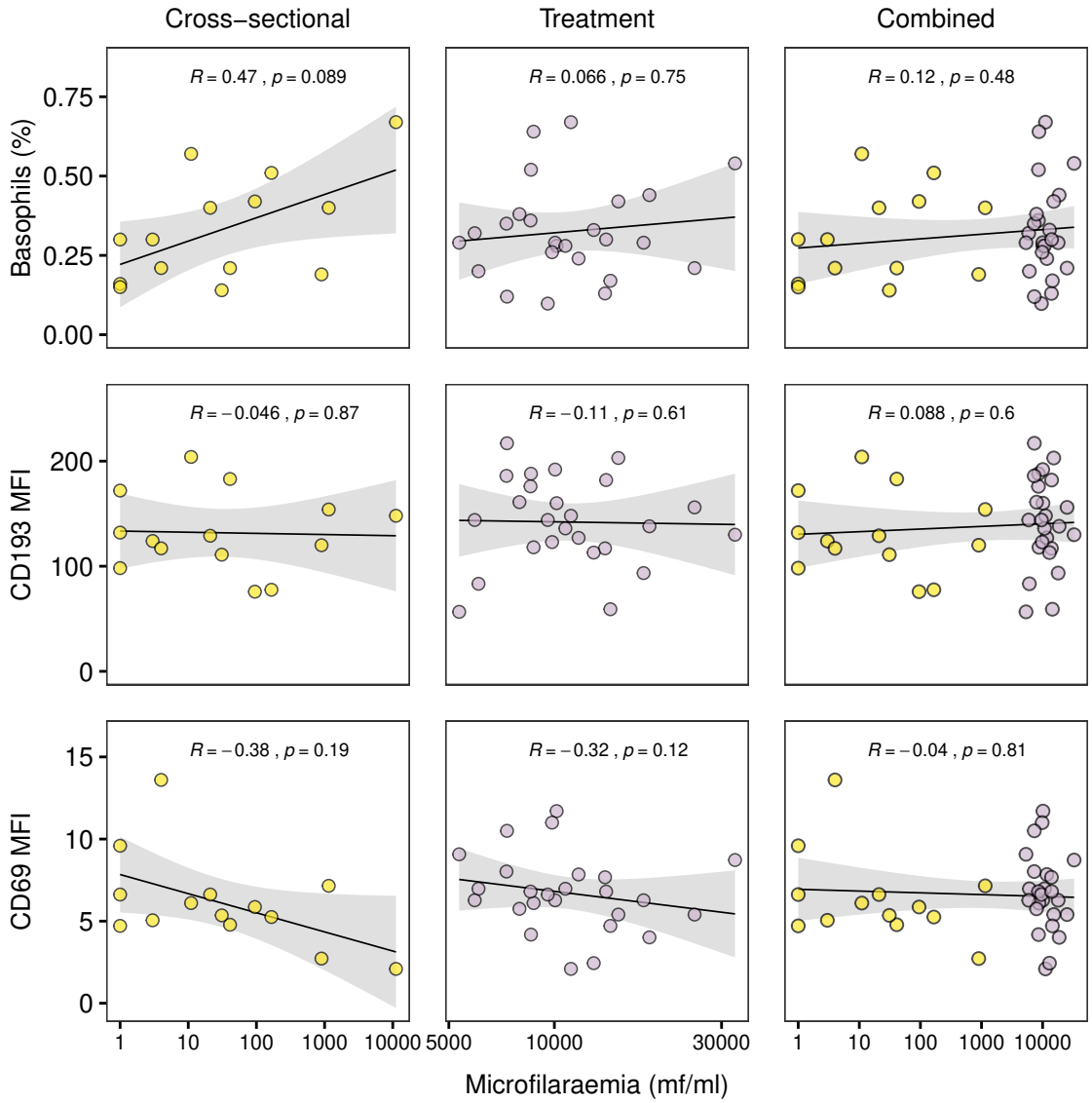

Figure 3.13: Correlation of basophil parameters and baseline microfilaraemia
Correlation of percentage of CD123+ CD193+ basophils among single leukocytes and expression of surface activation markers as median fluorescence intensity (MFI) and baseline *Loa loa*-microfilaraemia in 14 participants in the cross-sectional (left), 25 participants in the treatment study (middle) and the combined microfilaraemic population (right). Black lines and shaded areas represent linear regression lines with 95% confidence bands. R and p values were obtained by Spearman correlation analysis. Adapted from Burger et al.[235]

in the cross-sectional study, nor between baseline (6.8, 5.6–8.0), day 14 (6.1, 5.3–8.4) and end of follow-up (7.8, 5.8–10.2) in the treatment study (Figure 3.12).

3.3.3 Basophil parameters and microfilaraemia

Basophil numbers trended to correlate with microfilarial density in MF+ individuals in the cross-sectional (R = 0.47, p = 0.089), but not in the treatment study (R = 0.066, p = 0.75) or the pooled microfilaraemic population (R = 0.12, p = 0.48; Figure 3.13). There was no correlation between basophil activation markers and microfilaraemia in either of the three populations (Figure 3.13). No correlation was found between basophil parameters at day 14 and drop in microfilaraemia between baseline and day 14 as percentages of baseline in the treatment study (Figures S15–S17).

3.4 Myeloid-derived suppressor cells (MDSC)

3.4.1 MDSC numbers

PMN-MDSC numbers—i.e., percentage of CD66b+ CD11b+ CD14− PMN-MDSC among peripheral blood mononuclear cells—did not differ between MF+ (3.1%, 1.5–9.6), MF- (1.6%, 0.90–4.5) and LL- (2.8%, 0.34–7.4) participants in the cross-sectional study (Figure 3.14). In the treatment study, PMN-MDSC numbers did not differ between baseline (7.0%, 5.1–9.7), day 14 (7.6%, 3.0–13.2) and end of follow-up (6.5%, 4.6–13.2). M-MDSC numbers—as percentage of HLA-DR− CD14+ CD11b+ CD33+ M-MDSC among PBMC—did not differ between MF+ (0.36%, 0.13–0.46), MF- (0.21%, 0.16–0.34) and LL- (0.26%, 0.13–0.71) participants (Figure 3.14). However, upon treatment M-MDSC numbers trended to increase between baseline (0.36%, 0.22–0.54) and day 14 (0.57%, 0.19–1.2), and decrease until day 168 (0.26%, 0.15–0.54). PD-L1 expression of MDSC could not be analyzed due to unstable MFI of this parameter over time, that was observed upon quality control (Figure S18) and may be related to problems with the PD-L1 PE-Cy7 antibody.

3.4.2 MDSC and microfilaraemia

Neither PMN-MDSC nor M-MDSC numbers correlated with microfilaraemia in MF+ individuals in the cross-sectional, in the treatment study or the combined microfilaraemic population (Figure 3.15). No correlation was found between PMN-MDSC or M-MDSC numbers at day 14 and drop in microfilaraemia between baseline and day 14 as percentages of baseline in the treatment study (Figures S19–S20).

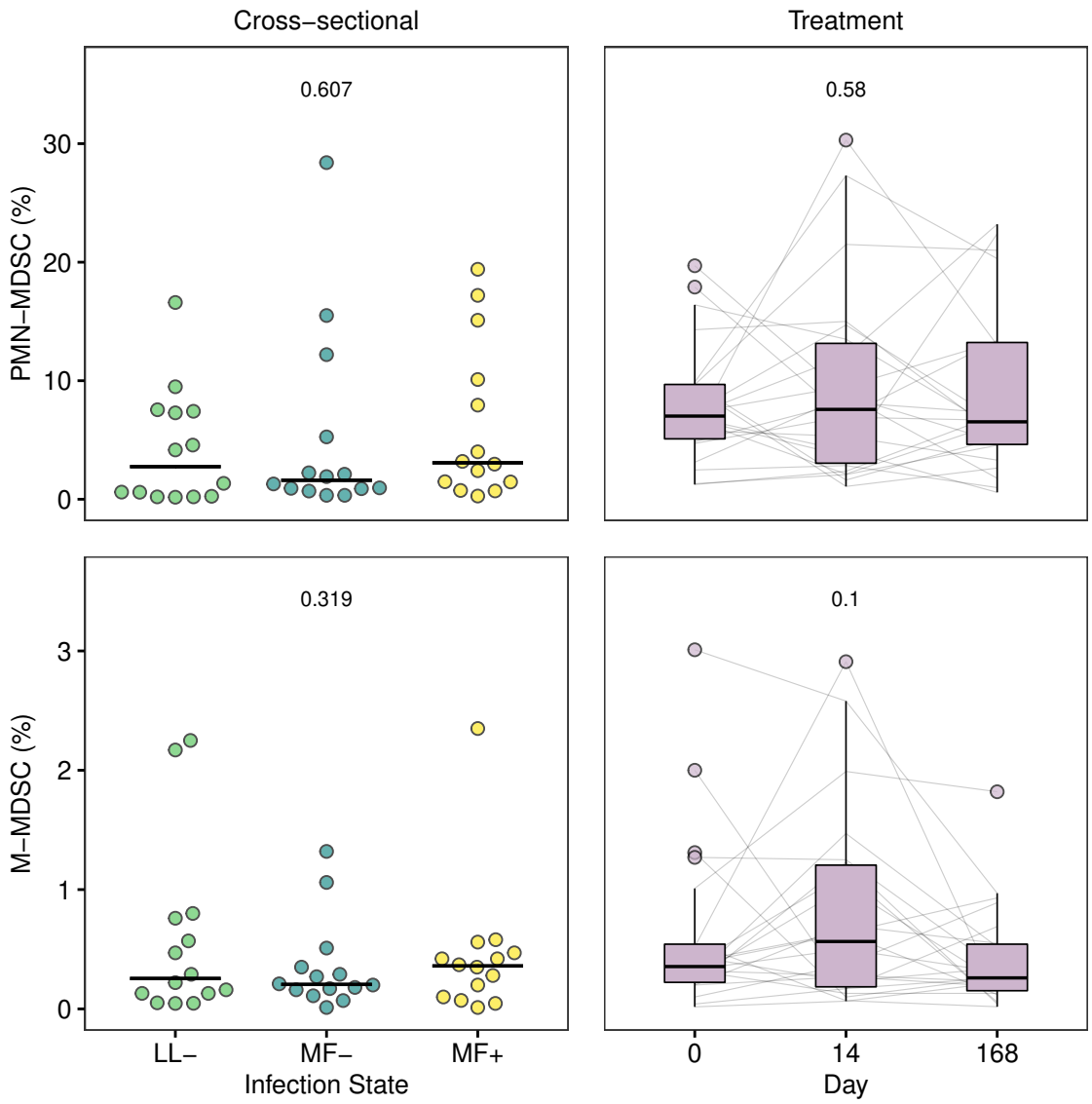

Figure 3.14: PMN-MDSC and M-MDSC numbers
Percentage of CD66b+ CD11b+ CD14− PMN-MDSC (top) and HLA-DR− CD14+ CD11b+ CD33+ M-MDSC (bottom) among single PBMC in 42 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left) and 22 microfilaraemic individuals upon treatment (right). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

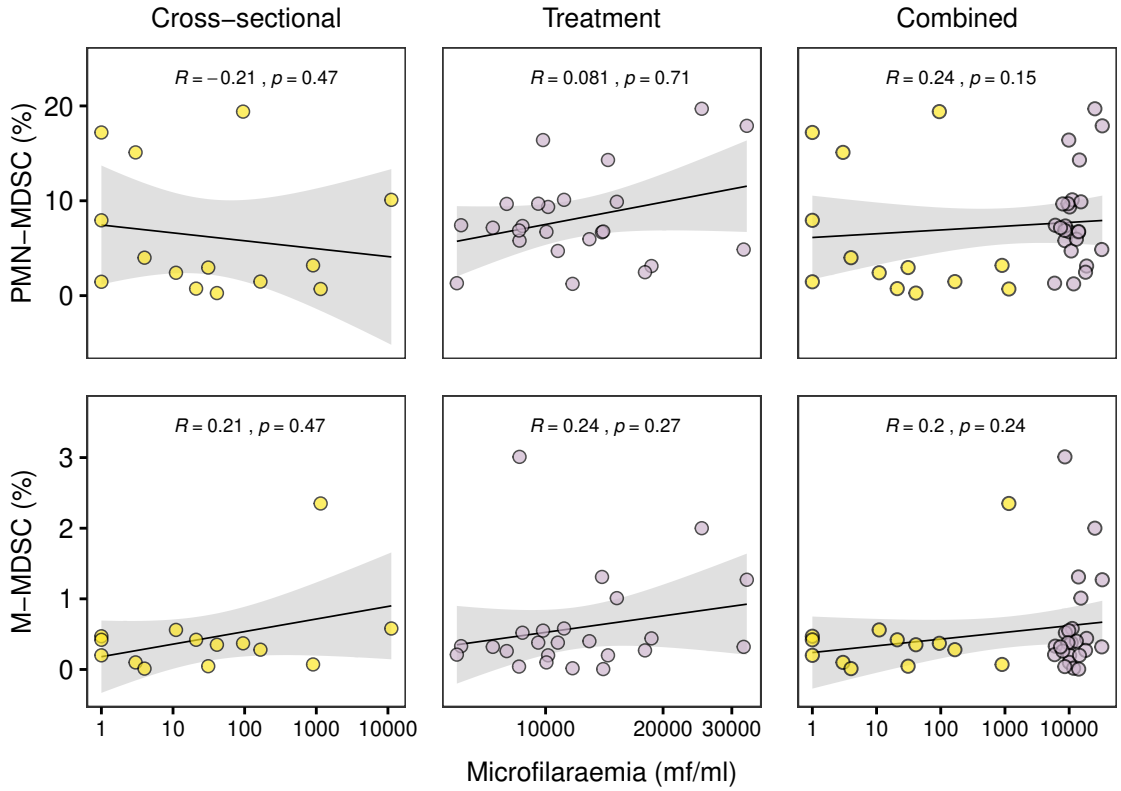

Figure 3.15: Correlation of MDSC numbers and baseline microfilaraemia
Correlation of percentage of CD66b+ CD11b+ CD14− PMN-MDSC and HLA-DR− CD14+ CD11b+ CD33+ M-MDSC among single PBMC and baseline *Loa loa*-microfilaraemia in 14 participants in the cross-sectional (left), 24 participants in the treatment study (middle) and the combined microfilaraemic population (right). Black lines and shaded areas represent linear regression lines with 95% confidence bands. R and p values were obtained by Spearman correlation analysis. Adapted from Burger et al.[235]

3.4.3 T cell proliferation-suppression assay for PMN-MDSC

T cell proliferation-suppression assays were conducted with PMN-MDSC isolated from five study participants—selected in a practical manner, i.e. by number of available PBMC—and donor PBMC from the same healthy individual in three independent experiments. Purity and viability of isolated CD66b+ PMN-MDSC were greater than 90% and 95% respectively, as assessed by flow cytometry. Participant PMN-MDSC showed a clear and dose-dependent suppression of both CD4+ and CD8+ healthy donor PBMC proliferation (Figure 3.16). Participant polymorphonuclear leukocyte controls did not impede CD4+ and CD8+ PBMC proliferation. An example of CFSE expression patterns and individual data of the five participants are given in Figures S21–S22.

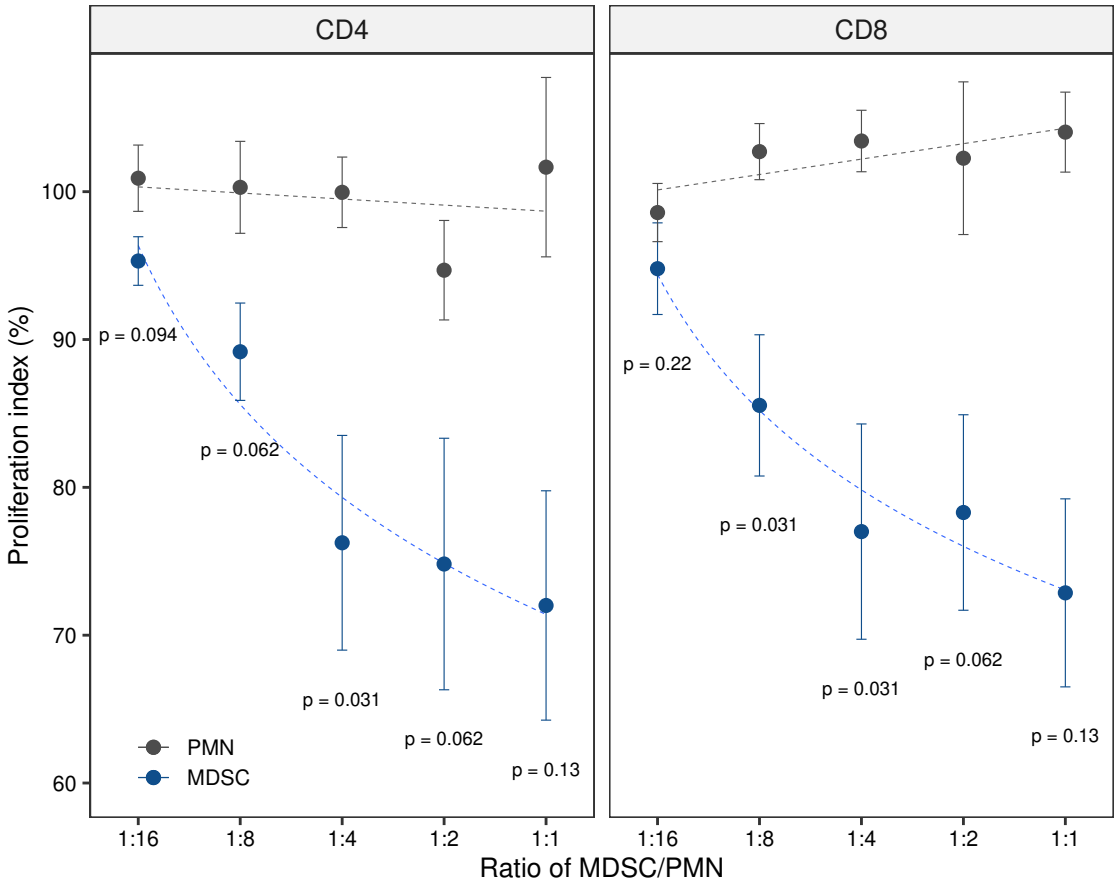

Figure 3.16: CD4 and CD8 T cell proliferation in the T cell suppression assay
Proliferation indices of CD4+ and CD8+ single live CFSE-stained healthy donor peripheral blood mononuclear cells (PBMC) after undergoing a four-day proliferation-suppression assay with increasing ratios of co-cultured polymorphonuclear myeloid-derived suppressor cells (MDSC, blue) or leukocyte controls (PMN, grey) from five study participants. Proliferation indices were normalized to the stimulated control (healthy donor PBMC only). Dots and error bars represent the mean and standard errors of the mean, respectively. One-sided p values were obtained by paired Wilcoxon signed-rank tests. Adapted from Burger et al.[235]

3.5 Circulating cytokines

Circulating cytokine levels could be measured by cytometric bead array in 33 and 21 participants in the cross-sectional and treatment study, respectively. IL-5 trended to higher levels in MF+ (1,460 pg/ml, 1,060–1,770) than MF- (1,030 pg/ml, 830–1,560) than LL- (400 pg/ml, 140–650) participants (Figure 3.17). In the treatment study, no significant changes were detected between baseline (780 pg/ml, 510–1,160) and day 14 (940 pg/ml, 540–1,960). However, after treatment IL-5 levels were significantly decreased to levels below baseline (450 pg/ml, 260–560). IL-10 levels did not differ between infection states in the cross-sectional study (Figure 3.17). Upon treatment IL-10 trended to increase between day 0 (1,160 pg/ml, 300–2,160) and day 14 (2,920 pg/ml, 820–3,530), followed by

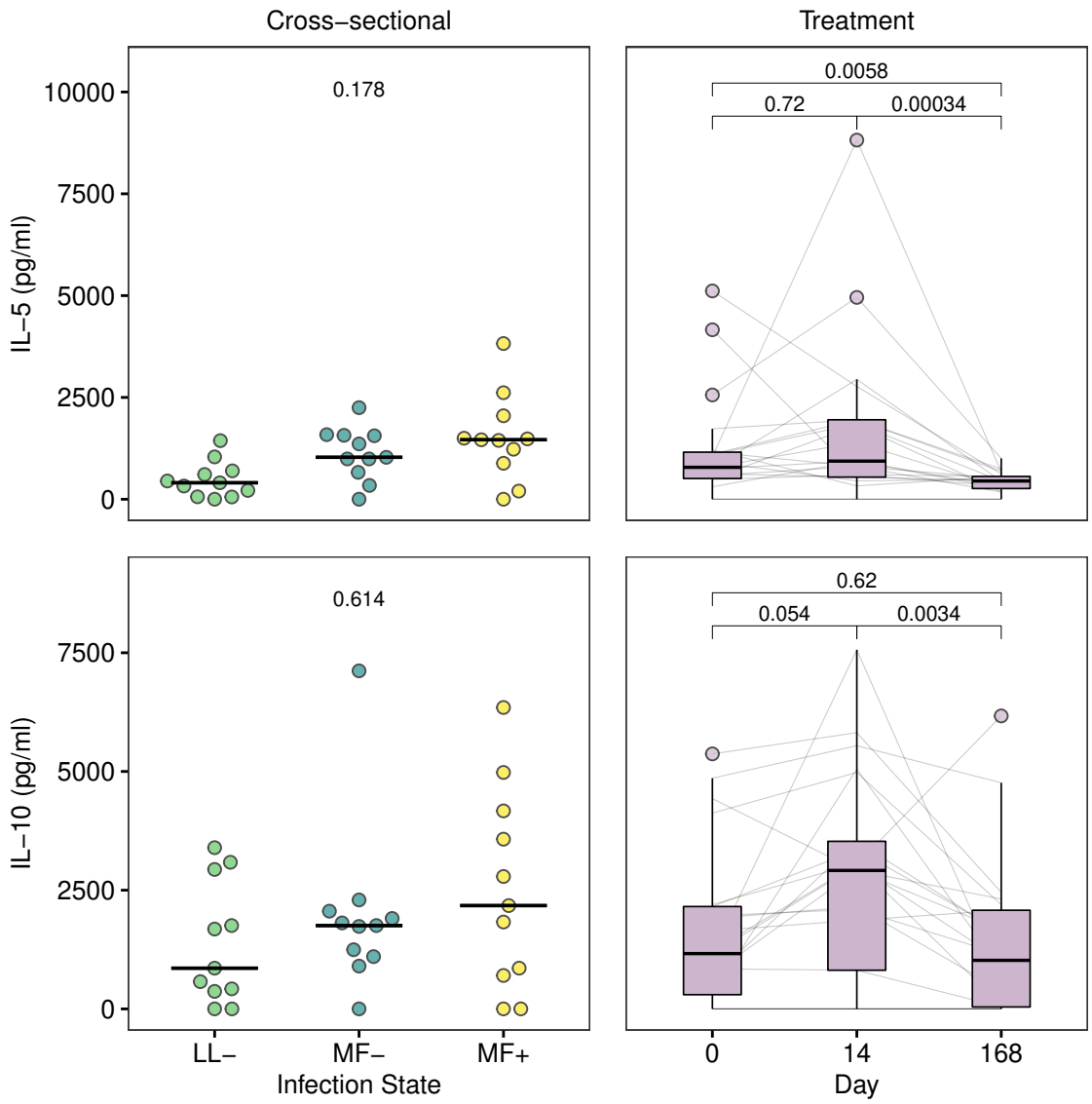

Figure 3.17: Circulating IL-5 and IL-10
Circulating interleukin-5 (IL-5, top) and IL-10 (bottom) levels in 33 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left) and 21 microfilaraemic individuals upon treatment (right). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Three and nine IL-5 measurements were below the lower limit of detection in the cross-sectional and treatment study, respectively. Adapted from Burger et al.[235]

a significant decrease by the end of follow-up (1,020 pg/ml, 40–2,080). Levels of IL-1b, IL-4, IL-6, GM-CSF, IFN-γ and TNF-α did not differ between infection states or upon treatment (Figures S23–S28).

4 Discussion

Chronic infection with *Loa loa* represents a remarkable immunological paradox: Microfilariae can circulate the bloodstream in densities over 100,000 mf/ml while adult worms migrate subcutaneously for decades, suggesting exemplary immunoregulation. However, once microfilaraemic individuals are treated they risk life-threatening complications most likely due to an overshooting inflammatory response upon rapid death of circulating microfilariae. Yet, most individuals remain amicrofilaraemic and distinct immunological differences have been assigned to microfilarial status. Here, we hypothesized that chronic *Loa loa* infection would be associated with an expansion of regulatory responses—particularly in microfilaraemic compared to amicrofilaraemic individuals—while treatment would trigger a pronounced inflammatory reaction spearheaded by eosinophils.

In apparent contrast with our hypothesis that chronic loiasis would primarily represent a distinct state of immunoregulation, we found a clear association of both microfilaraemic and amicrofilaraemic loiasis with peripheral blood eosinophilia and a trend towards higher eosinophil activation in MF+ than MF- than LL- individuals. Surprisingly, in this first ever investigation into MDSC in loiasis—although PMN-MDSC from *Loa loa*-infected individuals were functionally suppressive *in vitro*—we did not find increased MDSC numbers in *Loa loa*-infected individuals *in vivo*. In line with our hypothesis, treatment of microfilaraemic individuals led to a marked increase in eosinophil numbers and a distinct activated phenotype. After treatment, perturbations in eosinophil numbers and activation, as well as circulating IL-5 levels had declined to below baseline. In this first study assessing basophil numbers and activation in loiasis we found an increase in basophil numbers and decrease in activation after treatment, the implications whereof remain to be elucidated. Various aspects of the study warrant further consideration, as set out below.

4.1 Study population

4.1.1 Screening

We found a high prevalence of loiasis (47%, including both history of eye worm and microfilaraemia) in our screening population, which is in line with a large cross-sectional study recently conducted in the same area (51%).[5] Although we refrained from using more sensitive microscopy techniques (i.e. leukoconcentration) on a subset of participants (10%) that were ineligible for our immunological study, we found very similar rates of *Loa loa*-microfilaraemia (22%) as Veletzky et al. (24%).[5] A previous study did *not* apply concentration techniques and reported microfilaraemia in only 11% of individu-

als, which accordingly underestimated *Loa loa*-prevalence at only 31%.[73] These results illustrate why the choice of appropriate diagnostics for microfilaraemia is crucial in epidemiological studies investigating loiasis.[72] However, even when applying the currently best available diagnostics, *Loa loa*-prevalence might still be underestimated by microfilaraemia below the detection limit or adult filaria that do not migrate through the conjunctiva and thus are not reported as eye worm.

Here, individuals with history of eye worm or *Loa loa*-microfilaraemia were significantly older than the average screening population, which has been reported in numerous studies and presumably reflects the cumulative nature of *Loa loa*-infection.[5,72,80–83] In our study population, male sex was associated with higher rates of microfilaraemia. Although extensively described as a risk factor for *Loa loa*-microfilaraemia in epidemiological studies, research addressing the underlying behavioral risk factors (cumulative exposure level) and pathophysiology is still lacking.[5,72,73,76–79] Magnitude of microfilaraemia in our screening population was very low (63 mf/ml, IQR 6–909) compared to previous data from the same region (1,050 mf/ml, IQR 100–4,800), despite similar modes of sampling and diagnostics applied.[5]

4.1.2 Cross-sectional case-control study

In the cross-sectional study, we could not include the planned number of individuals due to several reasons: Firstly, malaria prevalence is high in the study population.[326] As immune perturbations caused by an active or recent malaria infection would have likely biased any investigations into *Loa loa*-induced immunomodulation, we had to exclude these individuals from our analysis.[337] Thus, 30% of our screening population were not eligible for the cross-sectional study based on their malaria RDT results. Secondly, *Loa loa*-infection correlates with age, while age can exert various effects on immune responses.[354,355] Therefore, our study population had to be age-matched across different age groups. This resulted in difficulties to include microfilaraemic and uninfected individuals in the youngest and oldest age group respectively. Although lower overall participant numbers might lead to increased probability of type II errors, the strict age-matching across many ages applied here ultimately increases the generalizability of our results.

4.1.3 Randomized controlled trial

In the treatment study, only 26 individuals receiving an albendazole-based treatment regimen were available for immunological analysis. This was mostly caused by consent withdrawal due to a high number of blood withdrawals necessary to track microfilaraemia, but also due to the complex setting—i.e. long transportation times and a highly mobile population—of the study area. Participants had a high prevalence of clinical signs of loia-

sis, exceeding the frequencies of the average *Loa loa*-infected population.[356] While this might simply be explained by chance, inclusion of participants from other studies who presented at the study center reporting *Loa loa*-symptoms and increased willingness of symptomatic individuals to participate in a treatment trial might have led to selection bias and a higher frequency of eye worm and other symptoms in the treatment population.

Only few prior studies have assessed the effect of albendazole on *Loa loa* microfilaraemia.[153–156] Here, we observed a more rapid decrease in microfilarial density than anticipated, with a reduction to below 50% of baseline within two days after initiation of treatment. This fast effect might be related to the higher dose of albendazole applied here,[153] while the overall more significant decrease of microfilaraemia might be explained by the longer duration of albendazole treatment in our study compared to other trials.[154–156] Moreover, the pharmacokinetic profile of albendazole varies extensively between individuals, which might be another reason for different effects observed across studies.[357] Given the prior publications above, albendazole is considered to have an embryostatic or embryotoxic effect, leading to a slow decrease in microfilaraemia due to impaired regeneration of microfilariae. However, this mode of action would not explain the fast decrease in microfilaraemia observed here, beginning right after the first day of treatment. It rather suggests that albendazole may have a microfilaricidal effect after all. Further research is needed to definitely understand the mode of action of albendazole against *Loa loa* microfilariae. Whereas continuation of albendazole treatment for another two weeks after the initial three-week cycle was associated with a further decrease in microfilaraemia, a similar effect could be reached with a single dose of ivermectin. However, our study might not have been adequately powered to detect differences between these two groups.

All treatment regimens were well tolerated, although we did not include individuals with very high microfilaraemia due to safety reasons and our study size did not allow to assess rare adverse events. Headache, diarrhea, fever and vertigo were the most common signs reported. Arthralgia, myalgia and pruritus are frequent AEs upon albendazole treatment of loiasis, but were highly present at baseline in our study population and thus often not reported as AEs.[153,155,156] While gastrointestinal symptoms like diarrhea have been frequently reported, we observed a higher proportion of neurological adverse events (headache, vertigo) usually associated with ivermectin or DEC.[148,155,156] This observation fits with the faster and stronger decrease in microfilaraemia in our study, which is considered to be associated with neurological (severe) AEs.[148] Moreover, AE incidence peaked around day 14 to 21 of albendazole treatment, which is later than after ivermectin (1–2 days) or DEC (8 hours).[148,149,171] This finding further underlines the close relationship between the decrease in microfilarial density and occurrence of adverse events upon

treatment of loiasis.

4.2 Eosinophils

Eosinophils are paramount effector cells in immune response to parasitic infections.[358] In loiasis, eosinophilia has been described in various case report series conducted at European and North American travel clinics, while evidence from endemic regions remains scarce.[91,95,96,98,99] In these series, eosinophilia was associated with a pro-inflammatory immune reaction and absence of microfilaraemia. Eosinophils are considered to play a crucial role in the immunopathogenesis of *Loa loa*-associated SAEs after treatment with DEC and ivermectin, however few studies have investigated eosinophil activation including surface activation markers in an endemic setting.[148] We hypothesized that both *Loa loa* infection *per se* as well as microfilaraemic compared to amicrofilaraemic loiasis would be associated with inhibition of eosinophil responses in terms of numbers and expression of surface activation markers. We further postulated that treatment of microfilaraemic loiasis would be associated with expansion and activation of the eosinophil compartment, followed by resolution of these immune perturbations after treatment.

4.2.1 Eosinophil numbers

In stark contrast to our hypothesis, both amicrofilaraemic and microfilaraemic loiasis were equally associated with marked eosinophilia in our cross-sectional study. Although eosinophilia has been extensively described in transient residents presenting at European and North American travel clinics, few studies have systematically investigated *Loa loa*-induced eosinophilia in endemic populations. Notably, an earlier study conducted in Gabon did not find a difference in eosinophil counts between *Loa loa*-infected and uninfected individuals.[359] However, no serologic testing (to detect circulating *Loa loa* SXP-1 IgG) was applied—meaning that *Loa loa*-infected individuals with microfilaraemia below the detection limit or adult filariae not migrating through the conjunctiva (thus not fulfilling eye worm criteria) could erroneously have been included in the control group.[235]

We hypothesized that microfilaraemic compared to amicrofilaraemic loiasis would be associated with an immunoregulatory response, allowing microfilariae to circulate the blood stream in high densities. Prior studies have suggested that microfilaraemia is associated with a dampened inflammatory response, particularly in the eosinophil compartment.[97,241,242] However, contrary to the differences in eosinophil numbers found between endemic and transient residents—where hyperresponsiveness is associated with eosinophilia and absence of microfilariae—we did not observe such an inverse association between microfilarial status and eosinophilia in our endemic population. In contrast, a

recent study from our center including over thousand individuals from a *Loa loa*-endemic region found a positive association of eosinophil numbers with not only *Loa loa*-infection *per se*, but also microfilaraemic compared to amicrofilaraemic loiasis; as well as a positive correlation between microfilarial loads and eosinophil counts.[356] Although we did not observe higher eosinophil counts in MF+ compared to MF- individuals—which might be explained by our smaller sample size or low microfilaraemia in the MF+ group—these results confirm that *Loa loa*-infection is associated with eosinophilia in endemic populations. Moreover, this clearly illustrates why data collected outside endemic areas (which includes most of the prior research concerning immunology in loiasis) need to be interpreted with care, underlining the importance of further research in endemic populations.

In accordance with our hypothesis, treatment of loiasis was associated with a marked increase in peripheral blood eosinophilia in our longitudinal study, which has been described in endemic and non-endemic populations.[148,170,360,361] Upon six-month follow-up, eosinophil numbers were dramatically decreased below baseline, indicating a significant long-term effect of treatment on the eosinophil compartment. This is in line with retrospective analyses of patients presenting at travel clinics in non-endemic areas, but the first study to demonstrate resolution of eosinophilia upon treatment of loiasis in an endemic population.[170,361]

4.2.2 Eosinophil activation markers

This is one of the first investigations to assess eosinophil surface activation markers in *Loa loa* infection. Similarly to the observed differences in eosinophil numbers, phenotypic activation patterns contradicted starkly with our starting hypothesis of (particularly microfilaraemic) *Loa loa* infection representing a primarily immunoregulatory state: In the cross-sectional study, eosinophils trended towards a more activated phenotype in MF+ than MF- than LL- participants, with higher expression of the activation marker CD123 and lower expression of the inverse activation marker CD193. These findings fit with the observations described above, suggesting that not only chronic *Loa loa* infection, but also presence of microfilaraemia is associated with expansion and activation of the eosinophil compartment. Upon treatment, eosinophils had decreased expression of the inverse activation marker CD125 and to some extent CD193 at day 14, suggesting not only increase in numbers but also activation of eosinophils upon clearance of microfilariae. At day 168 after treatment, CD123 expression was decreased significantly below baseline, while CD193 and to some extent CD125 increased after treatment. These results imply that after treatment of loiasis, eosinophils return to a less activated state, fitting with the decrease in eosinophil numbers observed here and elsewhere—thus confirming our initial hypothesis

that eosinophil-associated immune perturbations in *Loa loa*-infected individuals would resolve after treatment.[170]

While eosinophil expression of CD123, CD125 and CD193 followed a congruent pattern, the CD16 expression observed here was rather surprising.[235] Upregulation of surface CD16 in eosinophils has been shown in states of allergy and upon stimulation of eosinophils by pro-inflammatory cytokines as interferon gamma, implying a role in eosinophil activation.[235,283,284] However, in our study CD16 expression was *lower* in MF+ and MF- than LL- participants, was *increased* significantly after treatment at day 168 and correlated *inversely* with baseline microfilaraemia in the treatment study. These findings suggest that CD16 may play a different role in eosinophil activation than hypothesized by prior research. The implications hereof remain unclear, however, a recent publication proposing an immunomodulatory role of CD16+ eosinophils demonstrates that further research is needed to better understand eosinophil phenotypes and functions in general, but particularly in loiasis.[235,362]

In contrast to Herrick et al.—who reported CD69 upregulation in microfilaraemic individuals receiving ivermectin or DEC—we did not find any differences in CD69 expression between infection states or upon treatment.[148] This could be related to differences in the choice of anthelminthic agent or timing of sample collection.[235] Moreover, eosinophils have autofluorescent properties in the same wavelength as the fluorochrome we used to measure CD69 expression, potentially interfering with fluorescence emitted by CD69-bound antibodies and masking differences in CD69 expression.[363]

4.2.3 Associated circulating cytokines

Chronic *Loa loa* infection has been associated with an increased Th2-type immune response upon antigen stimulation and IL-5-dependent eosinophilia is considered to lead the hyperinflammatory responses associated with treatment-related SAEs.[148,228] We hypothesized that eosinophil-associated cytokine levels (i.e. IL-4, IL-5) would be decreased in microfilaraemic compared to amicrofilaraemic compared to *Loa loa*-uninfected individuals and increase in parallel to eosinophilia upon treatment of microfilaraemic individuals.

However, in the cross-sectional study we did not find any differences in circulating IL-5 or other cytokine levels between infection states. This seems to contradict results from a previous study that reported higher levels of Th2-type cytokines, including IL-5 in amicrofilaraemic compared to microfilaraemic individuals.[170] Although we may have been limited to detect these differences in cytokine levels by our small sample size and low microfilaraemia in the microfilaraemic group, studies including both transient and permanent residents have to be interpreted with care, as differences in immunological reactions

might not only be related to parasitological status, but heavily biased by underlying differences in the immune system of individuals primed by decades of parasitic infections (endemic residents) or not (transient residents). However, in another small study from an endemic population PBMC IL-5 production seemed to be higher in amicrofilaraemic than microfilaraemic participants upon antigen stimulation.[245] Thus, these cytokines should be further investigated in larger studies conducted in endemic areas.

In the treatment study, we did not find a significant difference in circulating IL-5 levels between baseline and upon treatment with albendazole at day 14. Previously, Herrick et al. reported a marked increase in IL-5 levels within one to three days after treatment with DEC and ivermectin, returning to levels around baseline at day seven. Although albendazole is considered to reduce microfilaraemia more slowly than DEC and ivermectin, we observed a more rapid decrease in microfilarial load in the first days of treatment than anticipated. Thus, it is possible that IL-5 levels had already decreased to values around baseline when we sampled for immunology at day 14. The role of IL-5 in sustaining eosinophilia in chronic *Loa loa* infections and triggering an increase in eosinophil numbers upon treatment was recently illustrated in a randomized controlled trial investigating the effect of a single-dose anti-IL-5 antibody (reslizumab) on post-treatment eosinophilia and AEs.[188] Eosinophil count was lowered significantly before treatment and post-DEC eosinophilia significantly reduced. However, reduced eosinophilia could not prevent post-treatment AEs in this small proof-of-principle study. In our study, IL-5 levels were decreased significantly below baseline after six months. This fits the significant decrease in eosinophilia after treatment described above, as well as results from a recent study.[170] In summary, these results underline the role of IL-5 in sustaining eosinophilia in chronic *Loa loa* infection.

4.3 Basophils

Basophils have emerged as important players in immune defense against helminth infections, mostly by initiating and sustaining type 2 immune responses, e.g. through release of significant quantities of IL-4—even outperforming CD4 T cells.[292–295] Yet, this is the first ever *in vivo* investigation of basophils in *Loa loa* infection to the author's knowledge.[235] As basophil functions are suppressed in some helminth infections, we hypothesized that chronic loiasis might be associated with decreased basophil numbers and/or phenotypic activation.[299,300] Due to their role in triggering anaphylactic reactions, we postulated that they might contribute to the inflammatory reactions observed upon treatment of microfilaraemic individuals. While we did not find any differences in basophil numbers or activation in the cross-sectional study, basophil numbers were significantly increased after treatment at day 168 compared to baseline. In apparent contrast, expression of the inverse

activation marker CD193 was higher at day 168 compared to baseline, suggesting reduced basophil activation after treatment. One could speculate whether an increase in basophil numbers after treatment may represent a sign of recovery from immune modulation by the parasite, as basophil functions seem to be suppressed in some helminth infections.[299,300] However, this would at first instance seem to contradict reduced basophil activation after treatment as implied by higher CD193 expression. Moreover, no differences have been found in basophil numbers between helminth-infected and -uninfected individuals here and elsewhere.[289] Thus, the precise role of basophils in *Loa loa*-infection remains to be further studied.

4.4 Myeloid-derived suppressor cells (MDSC)

4.4.1 MDSC numbers

In chronic loiasis, expansion of immunoregulatory pathways and T cell hyporesponsiveness have been described.[124,230–234,240] This immunomodulation seems to expand to bystander antigens, dampening immune responses to and increasing risk of co-infection with other pathogens.[228,237,238] MDSC expand in various chronic parasitic and filarial infections, modulating immune responses and potentially preventing parasite clearance.[310–313,315–320] Here, we hypothesized that MDSC contribute to immune regulation in chronic *Loa loa* infection.

However, in this first ever investigation into MDSC in loiasis we did not find any association of *Loa loa* infection and either PMN-MDSC or M-MDSC numbers in a cross-sectional setting. Upon treatment of MF+ individuals we observed no change in PMN-MDSC numbers, while M-MDSC trended to temporary increase during treatment with albendazole, decreasing to levels similar to baseline at day 168. This transient increase of M-MDSC during treatment most likely reflects a physiological anti-inflammatory response, balancing the general inflammatory response upon death of microfilariae and returning to the prior state after treatment.[235] Thus, in contradiction to our starting hypothesis we found no sign of participation of MDSC in the immunoregulatory response in chronic infection with *Loa loa*.

In a recent mouse model of filarial infection, MDSC expanded in the murine thoracic cavity (the site of infection with *Litomosoides sigmodontis*).[235,320] While it is challenging to assess tissue expansion of MDSC in *Loa loa*-infected human subjects (e.g. by skin biopsies during migration of adult filariae), a main site of microfilaraemic *Loa loa*-infection is indeed peripheral blood, where the highest parasite densities are reached. Although it might be possible that local, tissue-specific MDSC activation occurs, expansion into peripheral blood is common in both solid tumors and tissue-dwelling parasitic

infections.[235,303,364] Thus, although we could not assess tissue-specific MDSC, it seems unlikely that they would induce relevant immunosuppression while not expanding to peripheral blood.[235]

4.4.2 T cell proliferation-suppression assay

We demonstrated for the first time that PMN-MDSC isolated from a subset of *Loa loa*-infected individuals clearly suppress both CD4 and CD8 T cell proliferation in a proof-of-principle experiment *in vitro*.[235] However, we could not comprehensively quantify MDSC activation or function in our study participants. Although we included a marker of MDSC activation in our flow cytometry panel, PD-L1 expression could not be analyzed due to the unstable MFI of this parameter over time, that was retrospectively observed upon quality control (Figure S18) and may have been related to problems with the PD-L1 PE-Cy7 antibody. Quantifying T cell suppression of PMN-MDSC in all participants would have gone beyond the scope of this resource-limited pilot study of MDSC in loiasis. Thus, although the absence of expansion of MDSC populations in our study suggests that they are not key players in immunomodulation in *Loa loa* infection, we cannot completely rule out that they might contribute to immunoregulation by increased immunosuppressive activity, i.e. through cytokine release or upregulation of surface antigens as PD-L1. Therefore, future studies could investigate MDSC activation and function in this setting.[235]

4.4.3 Associated circulating cytokines

Filarial antigen drives the production of IL-10 in *Loa loa* infection, while MDSC modulate immune responses by secretion of IL-10.[230,305] We hypothesized that chronic *Loa loa* infection, but also microfilaraemic compared to amicrofilaraemic loiasis would be associated with expansion of regulatory responses and associated cytokines.

However, we did not find any differences in circulating IL-10 levels between infection states in the cross-sectional study. While IL-10 is produced upon antigen stimulation in *Loa loa*-infected individuals,[230] it remains unclear whether differences exist between microfilaraemic and amicrofilaraemic individuals: One early study from our center found an expansion of IL-10 expressing CD4 T cells in microfilaraemic compared to amicrofilaraemic subjects.[246] However, another study from Gabon found higher production of IL-10 upon antigen stimulation in amicrofilaraemic individuals.[245] A more recent investigation found no differences in either spontaneous or parasite-driven IL-10 production between microfilaraemic and amicrofilaraemic individuals.[230]

In the treatment study we observed a temporary increase of IL-10 at day 14 of albendazole treatment. Similar to the changes in M-MDSC numbers, this might be explained as

a physiological anti-inflammatory reaction accompanying a primarily pro-inflammatory response upon death of microfilariae, as described elsewhere.[148,235]

4.5 Limitations

One limitation of our study—besides small sample size—is the relatively low microfilaraemia of participants included in the cross-sectional study.[235] As age is an important confounder to control for in immunological studies and correlates with microfilaraemia in our study population, we needed to age-match participants and include a wide range of ages to avoid bias purely due to immunosenescence.[235] However, this led to relatively low microfilaraemia in our MF+ group, meaning that we might not have been able to detect differences in immunological profiles caused by presence of microfilaraemia instead of infection *per se*. However, we included at least some highly microfilaraemic individuals and found only few correlations between microfilaraemia and immunological parameters.

Another limitation is the rather low microfilaricidal activity of albendazole compared to drugs such as ivermectin or DEC. This suggests that we may have not been able to observe the most fulminant immune responses that could finally be responsible for life-threatening SAEs.[235] However, even after treatment with albendazole we observed both a substantial decrease in microfilaraemia and significant immunological responses, which may reflect the overshooting immune responses causing treatment-related SAEs in loiasis. Although our treatment population included individuals treated with ivermectin after a first cycle of albendazole, this subgroup was too small to allow for meaningful statistical comparisons.

Co-infection with other helminths is common in the study area and could potentially have biased our investigation into *Loa loa*-specific immune alterations.[327,328,365] In the cross-sectional study we did not assess helminth co-infections due to logistical reasons. However, we matched participants by sex, age and when possible, village to control for exposure so that any differences in helminth co-infections between the three groups should mostly be reduced to chance.[235] In the treatment study we assessed gastrointestinal helminth infection at baseline and found no differences in e.g. eosinophil numbers over the course of treatment between participants with and without positive stool microscopy, suggesting the effects observed are indeed related to *Loa loa*.[235]

Sex is an important determinant of immunological reactions and there are many sex-associated differences in loiasis, ranging from disease prevalence to risk of SAEs.[72,77,78,150] However, due to the explorative nature of our study we were limited in sample size and could not perform detailed subgroup analyses for co-factors (including sex, but also age) potentially associated with immunological differences in *Loa loa* infection, which should be a focus of future studies.

4.6 Conclusion

We demonstrated that both chronic infection with *Loa loa* and treatment of microfilaraemic loiasis are associated with eosinophilia and distinct patterns of eosinophil surface activation.[235] Based on prior research including permanent and transient residents of *Loa loa*-endemic areas we hypothesized that microfilaraemic loiasis would be associated with hyporesponsiveness in the eosinophil compartment. However, in our endemic population we found trends towards *higher* eosinophil activation in microfilaraemic subjects. In conjunction with a recent study from our center, that showed increased eosinophil numbers in microfilaraemic loiasis and a correlation between microfilarial load and eosinophil counts, we thus can refute that microfilaraemia is associated with reduced eosinophil activation.[356] Overturning this assessment has paramount implications: *Loa loa*-associated mortality correlates with microfilarial densities, while chronic eosinophilia can have devastating consequences, such as endomyocardial fibrosis and heart failure—that have been reported, but not systematically assessed in loiasis.[27,285] We thus postulate that chronic activation of eosinophils may play an important role in the immunopathogenesis of *Loa loa*-associated long-term morbidity and mortality.[235] Large longitudinal studies in endemic areas would be necessary to fully assess the causal effect of chronic eosinophilia on morbidity and mortality in loiasis.

Helminth parasites mastered immune evasion by millennia of co-evolution with their hosts. In chronic loiasis, the mechanisms that allow adult worms to migrate subcutaneously for decades and microfilariae to circulate the blood stream in enormous numbers without causing overt signs of acute systemic inflammation remain unidentified. Here, we hypothesized that MDSC would contribute to immunomodulation by *Loa loa*. Although we showed for the first time that PMN-MDSC isolated from *Loa loa*-infected individuals are functionally suppressive, our results do not support the hypothesis that MDSC contribute significantly to immune regulation in chronic loiasis. We thus posit that other potent mechanisms of immunomodulation must guarantee this exceptional parasite to evade effective host immune responses so successfully.[235] In one study from our center, T regulatory cells in cord blood from newborns correlated negatively with Th1 and Th17 cells.[235,240] Besides T regulatory cells, other mechanisms such as activation of B regulatory cells, alternatively activated macrophages, parasite antigen recognition and modulation of innate pattern-recognition receptors deserve further investigation.[235]

Unraveling the immunological pathways involved in treatment-related life-threatening adverse events, chronic immunomodulation by *Loa loa* and potentially long-term morbidity and mortality remains crucial to improve management of this *truly* neglected disease on both the individual and population level. Given the accumulating evidence of public health significance, it is long overdue to formally recognize loiasis as a neglected tropical

disease to generate more funding for targeted research and control programs, that have in the context of other NTDs been proven effective tools to alleviate significant disease burden in the most vulnerable populations. The ultimate goal should be elimination of all human filariases.

5 Summary

5.1 English summary

Loiasis is a neglected tropical disease caused by the filarial nematode *Loa loa*. It affects over 10 million people in Central and West Africa, where it causes significant morbidity and mortality. In chronic infection, microfilariae can circulate the blood stream in densities up to over 100,000 mf/ml without causing overt systemic inflammation, suggesting exemplary immunotolerance. However, once highly microfilaraemic individuals are treated, they risk life-threatening hyperinflammatory responses. Here, we hypothesized that chronic *Loa loa* infection—and microfilaraemic compared to amicrofilaraemic infection—would be associated with immunoregulation mediated by myeloid-derived suppressor cells, while treatment of microfilaraemic individuals would lead to an inflammatory response led by eosinophil and basophil activation. Thus, we investigated these innate immune cell subsets in two human studies in an endemic area in Gabon.

We included 42 sex- and age-matched *Loa loa*-microfilaraemic (MF+), -amicrofilaraemic (MF-) and uninfected (LL-) subjects in a cross-sectional case control study; as well as 26 MF+ individuals receiving different albendazole-based treatment regimens within a randomized controlled trial. Numbers and surface activation markers of eosinophils, basophils and MDSC subsets, as well as associated circulating cytokine levels were assessed by flow cytometry. A T cell proliferation-suppression assay was performed to confirm immunosuppressive capacity of isolated PMN-MDSC from *Loa loa*-infected participants.

Percentage of eosinophils was higher in *Loa loa*-infected than LL- participants (3.0%) but did not differ significantly between MF+ (13.1%) and MF- (12.3%) individuals. Upon treatment of MF+ subjects, we observed a marked increase in eosinophils between baseline (17.2%) and day 14 (24.8%) of treatment with albendazole, followed by a significant decrease to below baseline until day 168 (6.3%). Similar trends were observed for CD123 expression and circulating IL-5 levels, while opposite patterns evolved for the inverse activation markers CD193 and to some extent CD125. Basophil numbers did not differ between infection states in the cross-sectional study, but increased after treatment, just as basophil CD193 expression. While PMN-MDSC from *Loa loa*-infected participants were functionally suppressive, we found no difference in neither PMN-MDSC nor M-MDSC numbers between infection states or upon treatment.

In contrast to our hypothesis, we did not find evidence for involvement of PMN-MDSC or M-MDSC in immunomodulation by *Loa loa*, suggesting immune regulation through other pathways. We demonstrate that both chronic *Loa loa* infection as well as treatment of microfilaraemic loiasis are associated with marked eosinophilia

and distinct patterns of phenotypical activation markers that may contribute to inflammatory pathways in these settings. Further unraveling the immunological paradox of *Loa loa* infection will lead to improved management of this truly neglected disease.

5.2 German summary

Die Loiasis, aufgrund der gelegentlichen Migration erwachsener Würmer durch die Bindehaut auch als Afrikanischer Augenwurm bezeichnet, ist eine durch Filarien der Art *Loa loa* verursachte vernachlässigte Tropenkrankheit. Die Erkrankung betrifft über 10 Millionen Menschen in Zentral- und Westafrika, wo sie mit erheblicher Morbidität und Mortalität assoziiert ist. Bei der chronischen Erkrankung können Mikrofilarien in Konzentrationen von über 100.000 pro Milliliter im Blutkreislauf zirkulieren, ohne eine offensichtliche systemische Entzündungsreaktion zu verursachen. Dies lässt auf die Induktion einer beispielhaften Immuntoleranz schließen. Demgegenüber besteht das Risiko lebensbedrohlicher hyperinflammatorischer Reaktionen, wenn bei einer hochgradigen Mikrofilarämie eine Behandlung eingeleitet wird.

Im Rahmen dieser Arbeit wurde die Bedeutung verschiedener Zellarten der angeborenen Immunabwehr in der dichotomen Immunantwort auf *Loa loa*, in einer endemischen Region in Gabun untersucht. Zweiundvierzig nach Geschlecht und Alter gematchte *Loa loa*-mikrofilaräme (MF+), -amikrofilaräme (okkulte, MF-) und nicht infizierte (LL-) Proband*innen wurden in eine Querschnitts-Fall-Kontroll-Studie, sowie 26 MF+ Personen, die verschiedene Albendazol-basierte Behandlungsschemata im Rahmen einer randomisierten kontrollierten Studie erhielten, in eine Längsschnittstudie eingeschlossen. Der relative Anteil sowie die Oberflächenaktivierungsmarker von Eosinophilen, Basophilen und MDSC-Untergruppen wurden mittels Durchflusszytometrie analysiert, ebenso wie die zugehörigen Zytokinspiegel. Ein T-Zell-Proliferationssuppressionsversuch wurde durchgeführt, um die immunsuppressiven Kapazitäten von isolierten PMN-MDSC bei durch *Loa loa* infizierten Proband*innen zu demonstrieren.

Der Anteil der Eosinophilen war bei Teilnehmer*innen, die durch *Loa loa* infiziert waren höher als bei LL- Teilnehmer*innen (3,0%), unterschied sich jedoch nicht signifikant zwischen MF+ (13,1%) und MF- (12,3%) Proband*innen. Nach der Behandlung der MF+ Teilnehmer*innen konnte ein deutlicher Anstieg der Eosinophilen zwischen dem Ausgangswert (17,2%) und Tag 14 (24,8%) der Behandlung mit Albendazol beobachtet werden, gefolgt von einem signifikanten Rückgang unter den Ausgangswert bis Tag 168 (6,3%). Analog dazu konnten ähnliche Trends für die Expression von CD123 und die Spiegel von zirkulierendem Interleukin-5 (IL-5) beobachtet werden. Entgegengesetzte Muster zeigten sich hinsichtlich der inversen Aktivierungsmarker CD193 und in

gewissem Maße auch CD125. In der Querschnittsstudie konnte zwischen den Gruppen kein Unterschied in der Anzahl der Basophilen oder ihrer Expression von Aktivierungsmarkern festgestellt werden. Nach der Behandlung kam es jedoch zu einer Zunahme der Zirkulation, sowie der Expression von CD193 auf Basophilen. Obwohl PMN-MDSC von durch *Loa loa* infizierten Teilnehmer*innen funktionell suppressiv waren, konnte kein Unterschied in der Anzahl der PMN-MDSC oder M-MDSC zwischen den Gruppen der Querschnittsstudie oder nach Behandlung festgestellt werden.

Unsere Ergebnisse zeigen, dass sowohl die chronische Infektion mit *Loa loa* als auch die Behandlung der mikrofilarämen Loiasis mit einer signifikanten Eosinophilie und charakteristischen Expressionsmustern phänotypischer Aktivierungsmarker assoziiert sind. Diese könnten zu spezifischen proinflammatorischen Signalwegen in diesem Kontext beitragen. Obwohl PMN-MDSC von mit *Loa loa* infizierten Personen funktionell suppressiv waren, ergaben sich keine Hinweise für eine Beteiligung von PMN- oder M-MDSC an der Immunmodulation durch *Loa loa*. Dies lässt den Schluss zu, dass die Regulation des Immunsystems bei der chronischen Loiasis über andere Mechanismen erfolgt. Eine weitere Entschlüsselung des immunologischen Paradoxons der chronischen Loiasis könnte zu einem verbesserten Management dieser vernachlässigten Krankheit auf individueller und bevölkerungsbezogener Ebene führen.

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7 Declaration of contributions

This work was supervised by Prof. Dr. Dr. Ayola Akim Adegnika and Dr. Matthew BB McCall at the Centre de Recherches Médicales de Lambaréné and the Institute of Tropical Medicine, University of Tübingen. The immunological study was designed by Dr. Matthew BB McCall. The underlying cross-sectional study was designed by Dr. Luzia Veletzky, Dr. Wolfram Metzger, Prof. Dr. Benjamin Mordmüller and Prof. Dr. Michael Ramharter. The randomized controlled trial was designed by Dr. Rella Zoleko-Manego, Prof. Dr. Peter G Kremsner, Prof. Dr. Ghyslain Mombo-Ngoma and Prof. Dr. Michael Ramharter.

The screening activities, the immunological part of the cross-sectional study and the randomized controlled trial, as described in Sections 2.2–2.3, were conducted by Dr. Rella Zoleko-Manego, Ruth Kreuzmair, Wilfrid Ndzebe Ndoumba, the doctoral candidate, Dr. Rafiou Adamou, Dorothea Ekoka Mbassi, Dr. Dearie Glory Okwu and Lia-Betty Dimessa Mbadinga under the supervision of Dr. Luzia Veletzky, Dr. Wolfram Metzger, Prof. Dr. Benjamin Mordmüller, Prof. Dr. Michael Ramharter, Prof. Dr. Ghyslain Mombo-Ngoma, Prof. Dr. Dr. Ayola Akim Adegnika and Dr. Matthew BB McCall.

The doctoral candidate established all immunological assays (Sections 2.4–2.5) at CERMEL under the supervision of Dr. Matthew BB McCall. The assays described in Sections 2.5.2 and 2.5.3 were developed by the doctoral candidate based on protocols established by Dr. Carlos Lamsfus Calle at the Institute of Tropical Medicine in Tübingen, who gave technical advice during the implementation phase at CERMEL. The experiments outlined in the finalized protocols in Sections 2.5.1–2.5.3 were conducted by the doctoral candidate and Dr. Rafiou Adamou. The experiments described in Section 2.5.4 were conducted by Dr. Rafiou Adamou, Anne Marie Nkoma Mouima, Carole Mamgno Tabopda, Roukoyath Moyoriola Adegnika and Ayong More. The doctoral candidate and Dr. Rafiou Adamou curated the data. The doctoral candidate independently conducted the selection of statistical methods, formal statistical analysis and all data visualization under the supervision of Dr. Matthew BB McCall.

Dr. Julian Matthewman, Hannah Kaip, Dr. Erik Koehne, Franca Burger and Dr. Karola Schiele provided proofreading for orthography and grammar. Klara Koch offered technical guidance in the design of Figure 2.2. The doctoral candidate affirms that he has written the thesis independently under the supervision of Prof. Dr. Dr. Ayola Akim Adegnika and Dr. Matthew BB McCall and that he has not used any other sources than those indicated.

Tübingen, August 17, 2024

8 Publications

Burger G, Adamou R, Kreuzmair R, Ndzebe Ndoumba W, Ekoka Mbassi D, Mouima AMN, Tabopda CM, Adegnika RM, More A, Okwu DG, Mbadinga LD, Lamsfus Calle C, Veletzky L, Metzger WG, Mordmüller B, Ramharter M, Mombo-Ngoma G, Adegnika AA, Zoleko-Manego R, McCall MBB. *Eosinophils, basophils and myeloid-derived suppressor cells in chronic Loa loa infection and its treatment in an endemic setting*. PLoS Neglected Tropical Diseases. 2024 May 21;18(5):e0012203.
DOI: 10.1371/journal.pntd.0012203.

Veletzky L, Eberhardt KA, Hergeth J, Stelzl DR, Zoleko Manego R, Kreuzmair R, **Burger G**, Mischlinger J, McCall MBB, Mombo-Ngoma G, Adegnika AA, Agnandji ST, Matsiegui PB, Lell B, Kremsner P, Mordmüller B, Tappe D, Ramharter M. *Analysis of diagnostic test outcomes in a large loiasis cohort from an endemic region: Serological tests are often false negative in hyper-microfilaremic infections*. PLoS Neglected Tropical Diseases. 2024 Mar 14;18(3):e0012054. DOI: 10.1371/journal.pntd.0012054.

Veletzky L, Eberhardt KA, Hergeth J, Stelzl DR, Zoleko Manego R, Mombo-Ngoma G, Kreuzmair R, **Burger G**, Adegnika AA, Agnandji ST, Matsiegui PB, Boussinesq M, Mordmüller B, Ramharter M. *Distinct loiasis infection states and associated clinical and hematological manifestations in patients from Gabon*. PLoS Neglected Tropical Diseases. 2022 Sep 19;16(9):e0010793. DOI: 10.1371/journal.pntd.0010793.

Interim results of this research project were presented at the 29th Annual Meeting of the German Society for Parasitology and awarded with a first prize for best talk:

Burger GM, Adamou R, Kreuzmair R, Ndzebe Ndoumba W, Veletzky L, Metzger WG, Mordmüller B, Ramharter M, Mombo-Ngoma G, Adegnika AA, Zoleko-Manego R, McCall MBB (2021, March 15–17). *Eosinophil activation in response to treatment of chronic Loiasis* [Oral conference presentation]. 29th Annual Meeting of the German Society for Parasitology, Bonn, Germany.

Other publications that may follow from this work and are not yet published at the time of writing will be available via the author's Open Researcher and Contributor ID (ORCiD):

0000-0002-0142-5284

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I am truly humbled by the support of my co-supervisor, Dr. Matthew BB McCall. He deserves my deepest gratitude, not only for teaching me in the lab, supervising the project and greatly improving the quality of this manuscript through his excellent advice; but most importantly for being an inspiring mentor, always available when needed and generously sharing his (truly infectious) passion for science and exquisite sense of humor.

I am very grateful to Dr. Rafiou Adamou for his tireless work in the lab, for his good company when we had to process samples late at night, for continuing the experiments after my departure and for many fruitful scientific discussions.

I would like to acknowledge the entire outstanding scientific team: Ruth Kreuzmair, Dr. Wilfrid Ndzebe Ndoumba, Dorothea Ekoka Mbassi, Anne Marie Nkoma Mouima, Carole Mamgno Tabopda, Roukoyath Moyoriola Adegnika, Ayong More, Dr. Dearie Glory Okwu, Lia-Betty Dimessa Mbadinga, Dr. Carlos Lamsfus Calle, Dr. Luzia Veletzky, Dr. Wolfram Metzger, Prof. Dr. Benjamin Mordmüller, Prof. Dr. Michael Ramharter, Prof. Dr. Ghyslain Mombo-Ngoma and Dr. Rella Zoleko-Manego, as well as Ebe Suzanne Mengue, Roger Mouckoga Moussodou, Val Verlin Balouka and all the nurses, field workers, drivers, logisticians and administrative staff at CERMEL who contributed to this project. I am deeply grateful to the population of Estuaire, Moyen-Ogooué and Ngounié for their trust and participation in our research. I would like to sincerely thank Dr. Julian Matthewman, Hannah Kaip, Dr. Erik Koehne, Franca Burger, Dr. Karola Schiele, Klara Koch, Georg Schweizer, Dr. Hannes Becker, Louisa Silberkuhl and Christoph Jans for their valuable support and advice during the writing process.

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10 Annex

10.1 Supplementary Figures

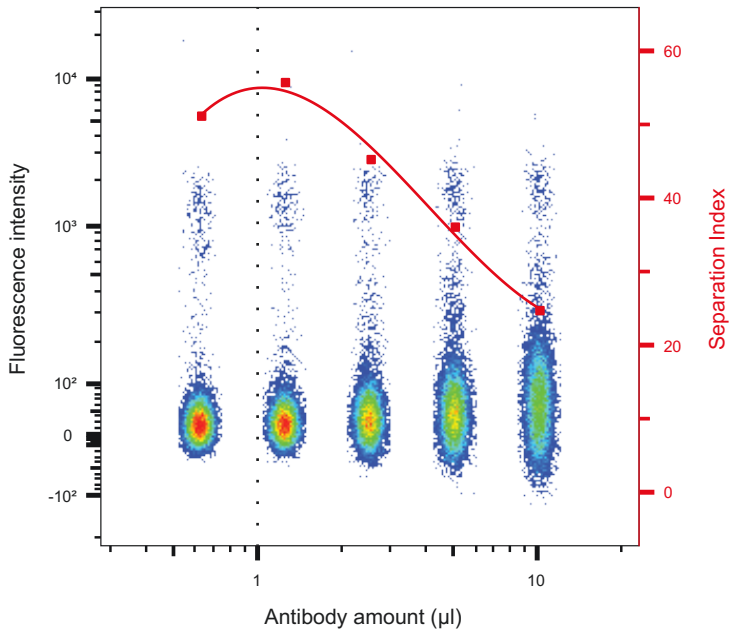

Figure S1: Antibody titration for flow cytometry
Fluorescence intensity of stained and unstained populations for different antibody amounts of CD193 PerCP-Cy5.5. Separation indices (SIs, red squares) were calculated as described in Equation 1. The red line represents the 3^{rd} degree polynomial regression line of the SIs. A volume of 1 μl was used in further experiments for optimal separation.

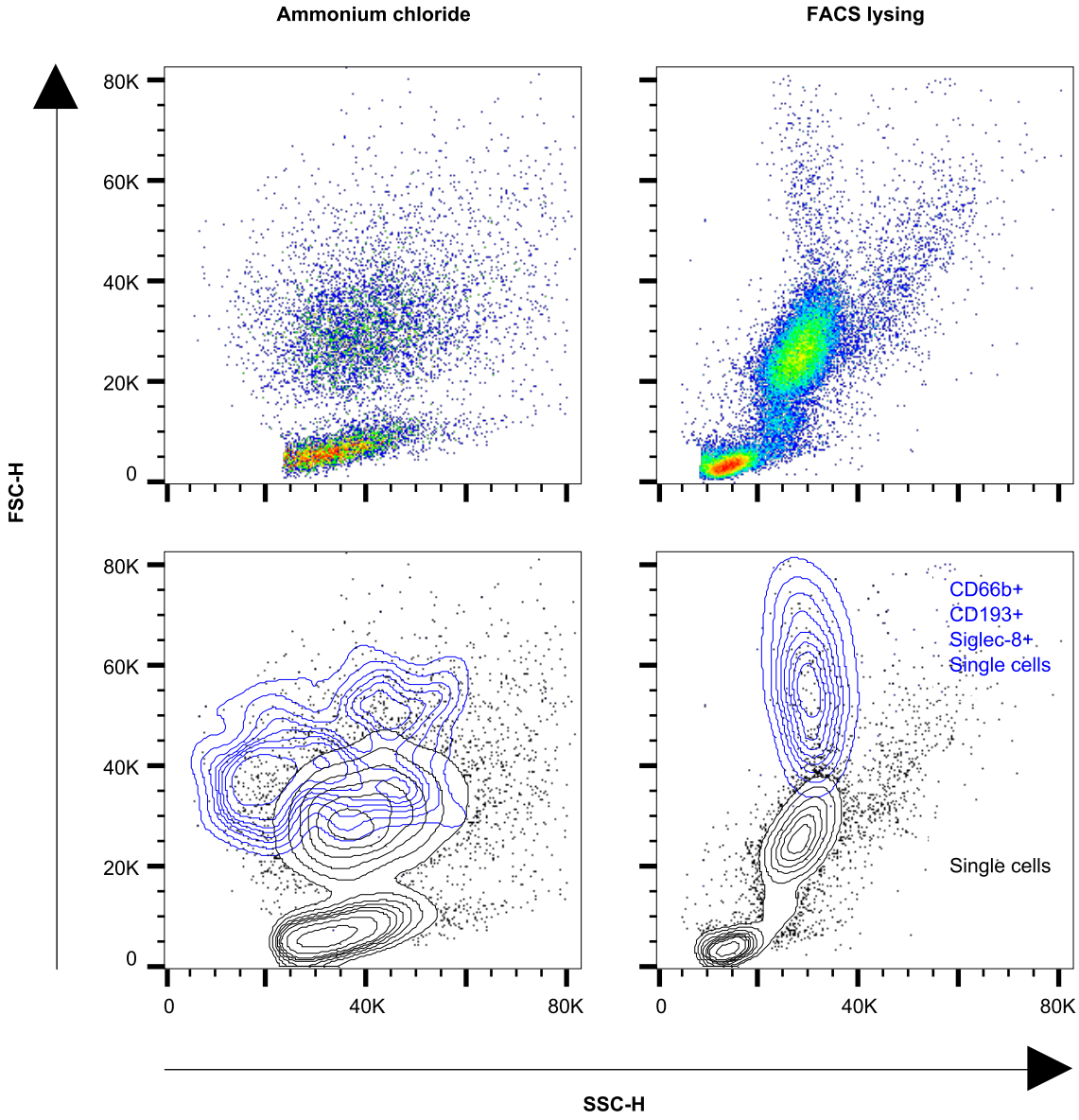

Figure S2: Red blood cell lysis buffer comparison
Scatter plots of single leukocytes from the same healthy donor after red blood cell lysis with either ammonium chloride (left) or FACS lysing solution (right). The lower plots show the overlay of single leukocytes (black) and CD66b+ CD193+ Siglec-8+ eosinophils (blue).

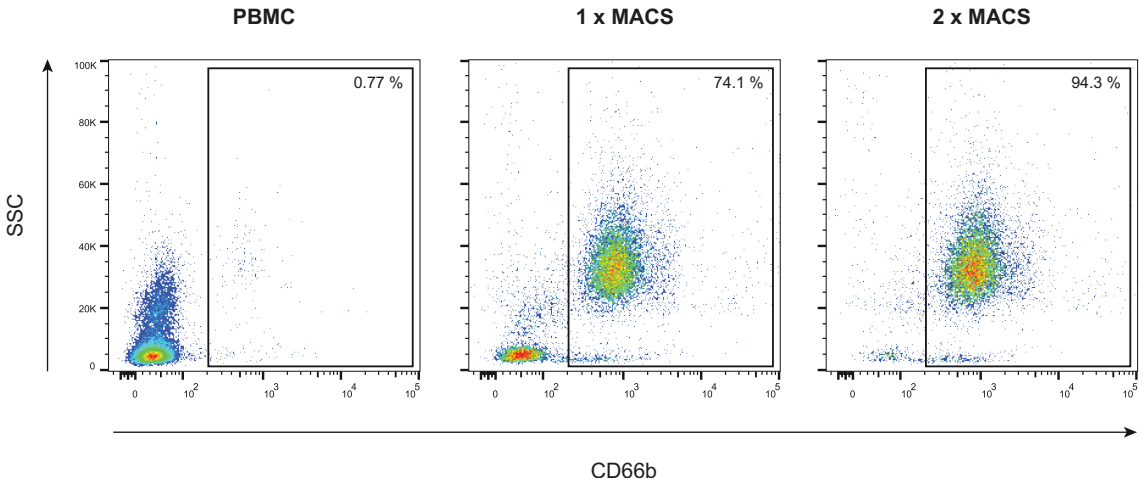

Figure S3: PMN-MDSC isolation by MACS
PBMC from a healthy volunteer were stained with CD66b and incubated with anti-FITC microbeads. PMN-MDSC were isolated using either one or two consecutive isolation steps with MS MACS columns. PBMC and isolated PMN-MDSC were stained with propidium iodide and acquired by flow cytometry. Single cells showed good viability ($\geq$ 95%) in all conditions. Two-step MACS isolation achieved preferable purity (percentage CD66b+ among single live cells).

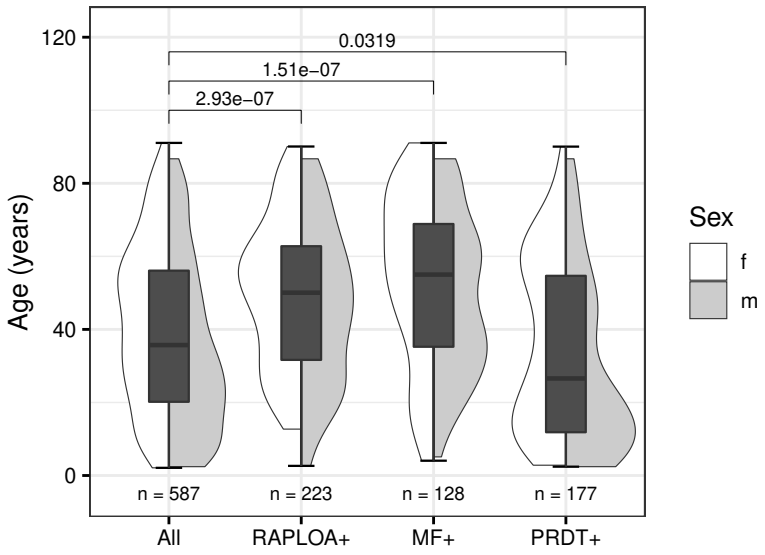

Figure S4: Age related to diagnostic criteria in the screening population
Splitted violin plots represent the relative age distribution for female and male individuals. RAPLOA+: Participants with positive RAPLOA results. MF+: Microfilaraemic participants. PRDT+: Participants with positive *Plasmodium spp.* RDT. Exact age was not available for 18 participants.

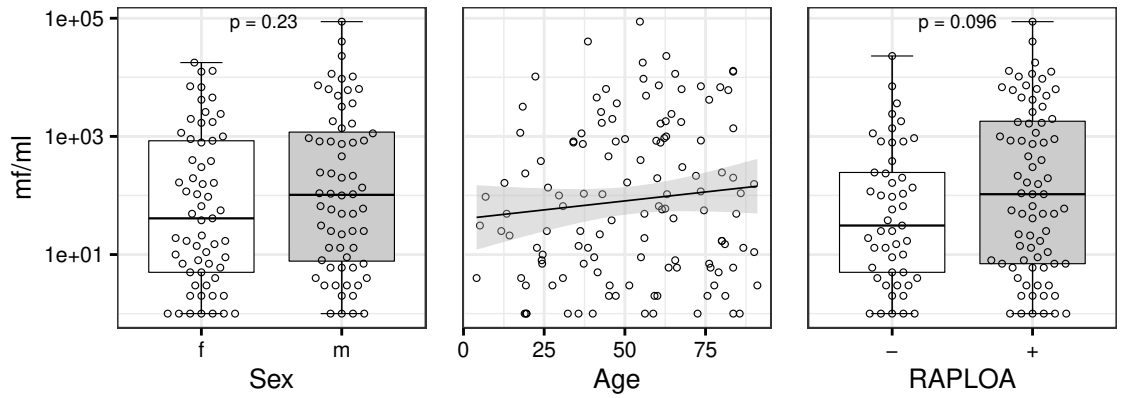

Figure S5: *Loa loa* microfilaraemia by characteristics of the screening population
All screened participants with microscopically diagnosed *Loa loa* microfilaraemia and complete screening data (sex, age, RAPLOA, n=125) were included in the analysis. Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR.

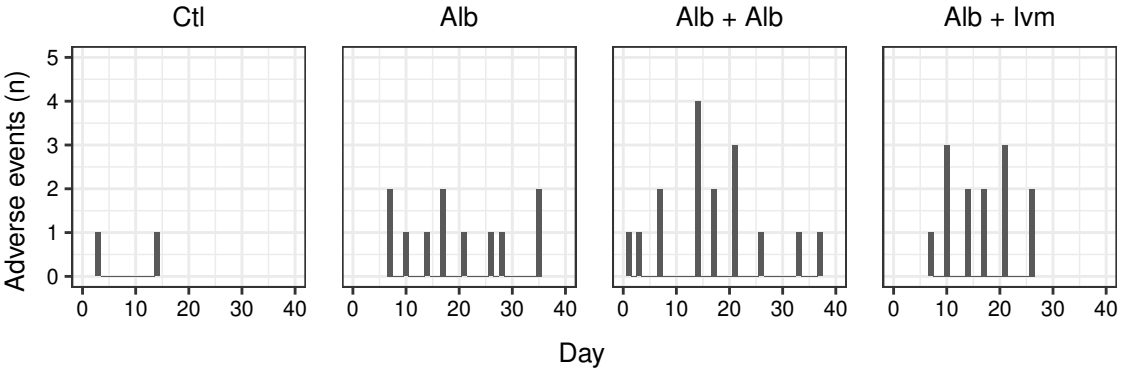

Figure S6: Timing of adverse events by treatment group
Number of adverse events by day of recording and treatment group in the first 40 study days.

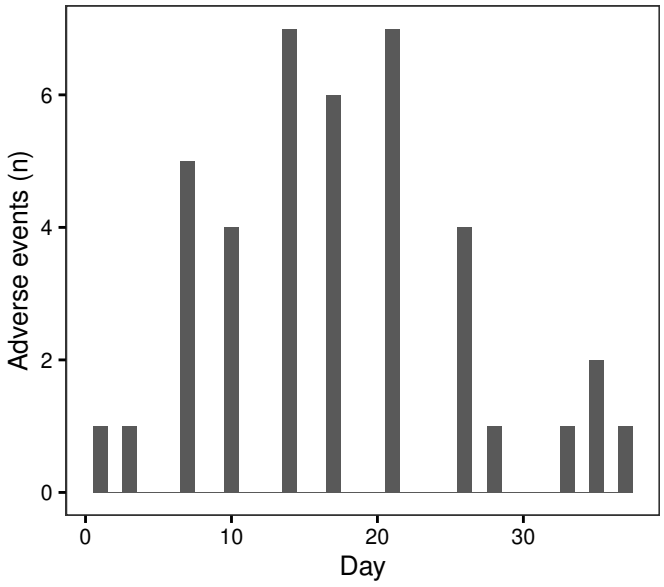

Figure S7: Timing of adverse events in treated individuals
Number of adverse events by day of recording in treated individuals (group 2–4) in the first 40 study days.

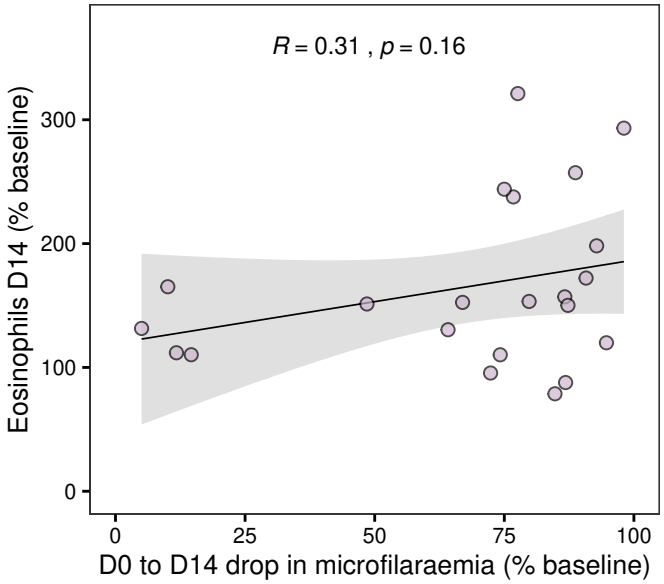

Figure S8: Correlation of change in microfilaraemia and eosinophil numbers
Correlation of percentage of CD125+ CD193+ eosinophils among single leukocytes and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

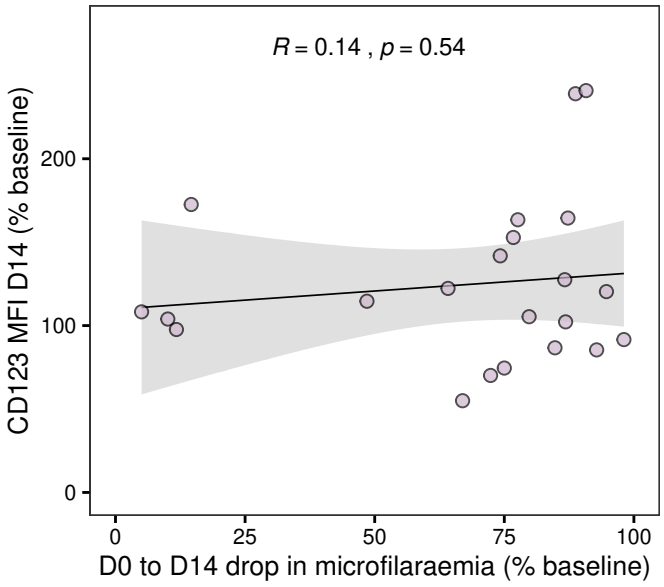

Figure S9: Correlation of change in microfilaraemia and CD123 expression of eosinophils
Correlation of CD123 MFI of CD125+ CD193+ eosinophils and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

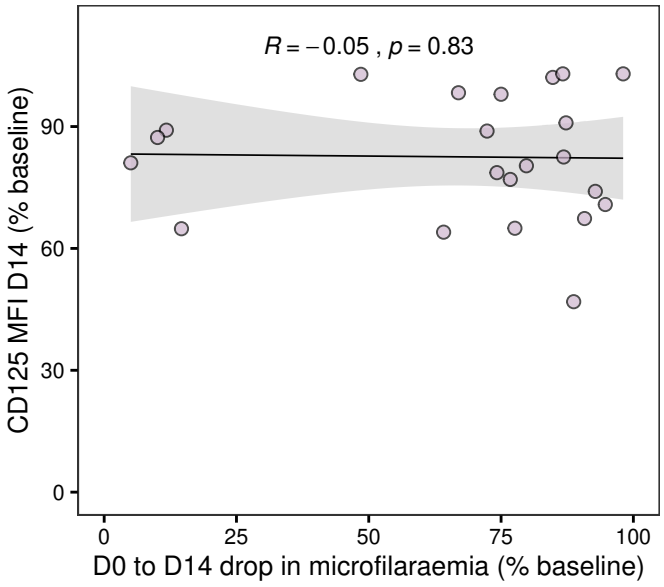

Figure S10: Correlation of change in microfilaraemia and CD125 expression of eosinophils
Correlation of CD125 MFI of CD125+ CD193+ eosinophils and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

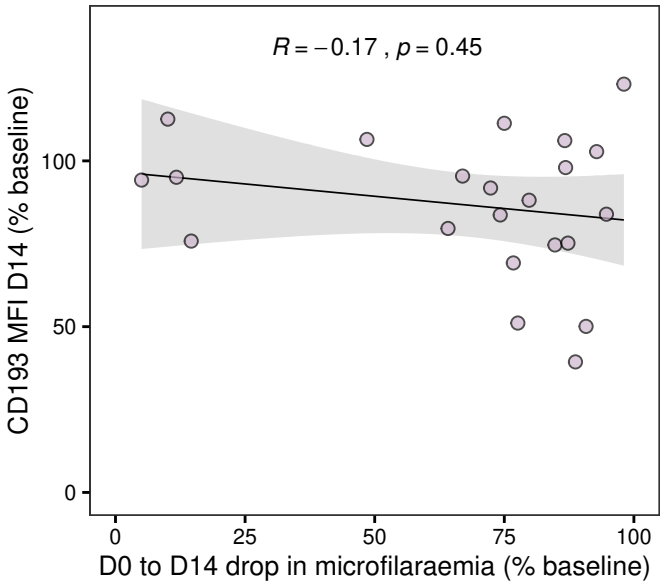

Figure S11: Correlation of change in microfilaraemia and CD193 expression of eosinophils Correlation of CD193 MFI of CD125+ CD193+ eosinophils and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

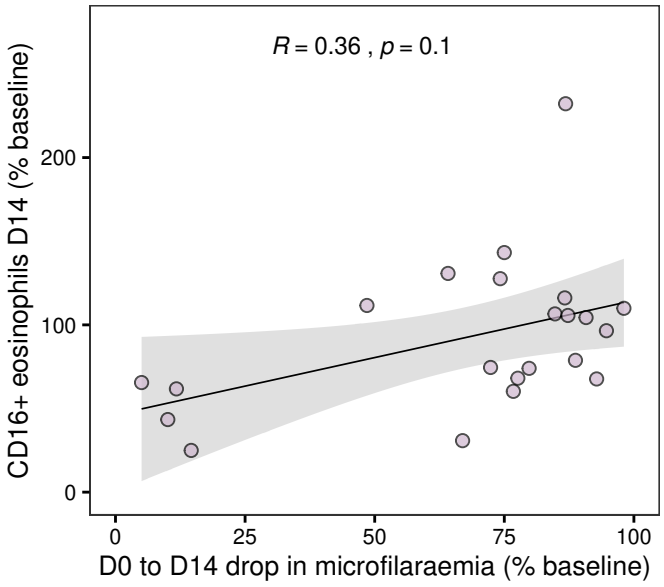

Figure S12: Correlation of change in microfilaraemia and CD16 expression of eosinophils Correlation of percentage of CD125+ CD193+ eosinophils expressing CD16 and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

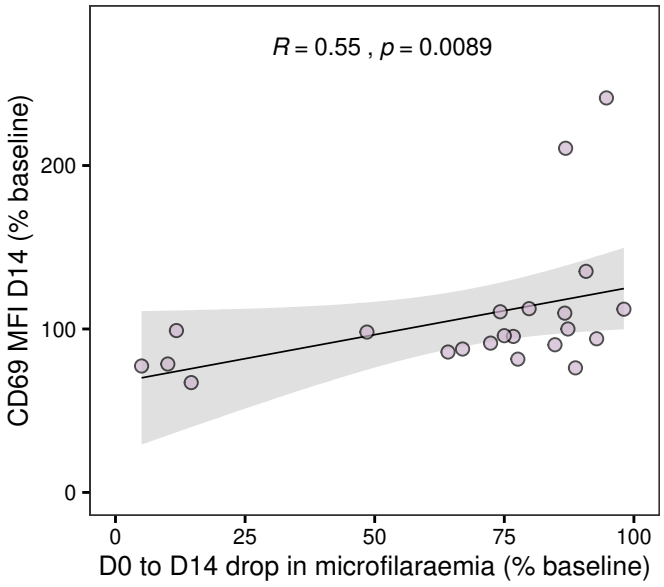

Figure S13: Correlation of change in microfilaraemia and CD69 expression of eosinophils
Correlation of CD69 MFI of CD125+ CD193+ eosinophils and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

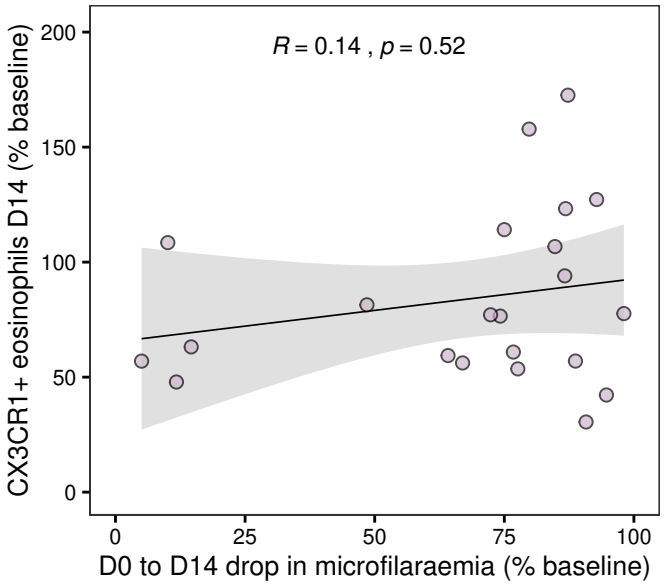

Figure S14: Correlation of change in microfilaraemia and CX3CR1 expression of eosinophils
Correlation of percentage of CD125+ CD193+ eosinophils expressing CX3CR1 and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

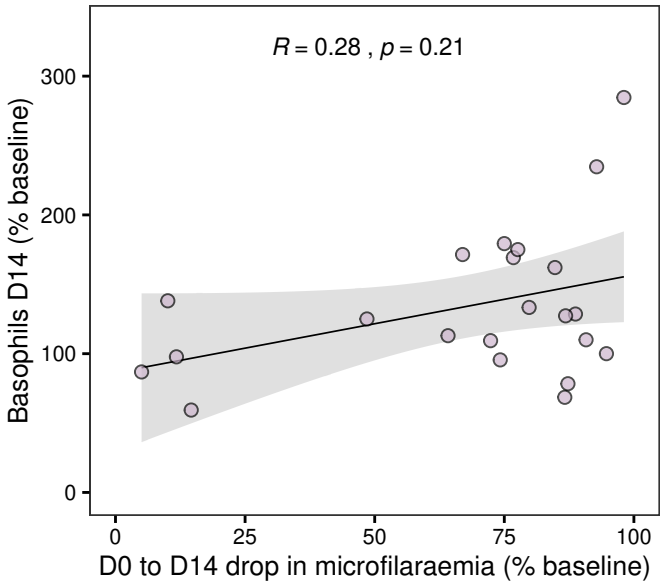

Figure S15: Correlation of change in microfilaraemia and basophil numbers
Correlation of percentage of CD123+ CD193+ basophils among single leukocytes and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

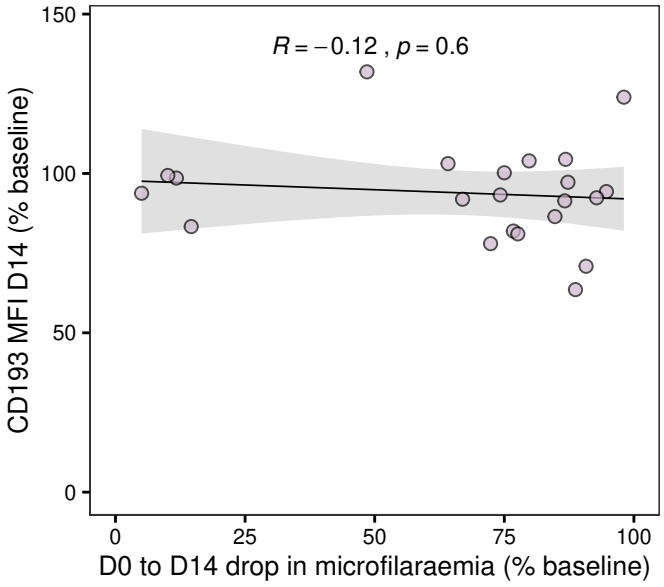

Figure S16: Correlation of change in microfilaraemia and CD193 expression of basophils
Correlation of CD193 MFI of CD123+ CD193+ basophils and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

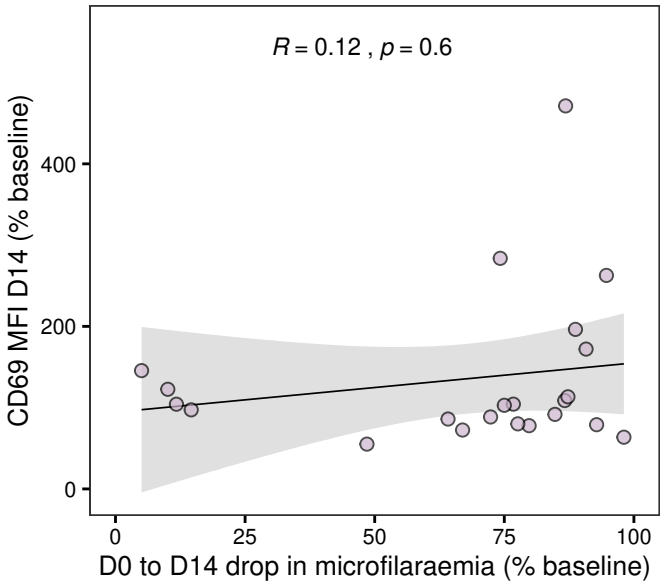

Figure S17: Correlation of change in microfilaraemia and CD69 expression of basophils
Correlation of CD69 MFI of CD123+ CD193+ basophils and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4). Adapted from Burger et al.[235]

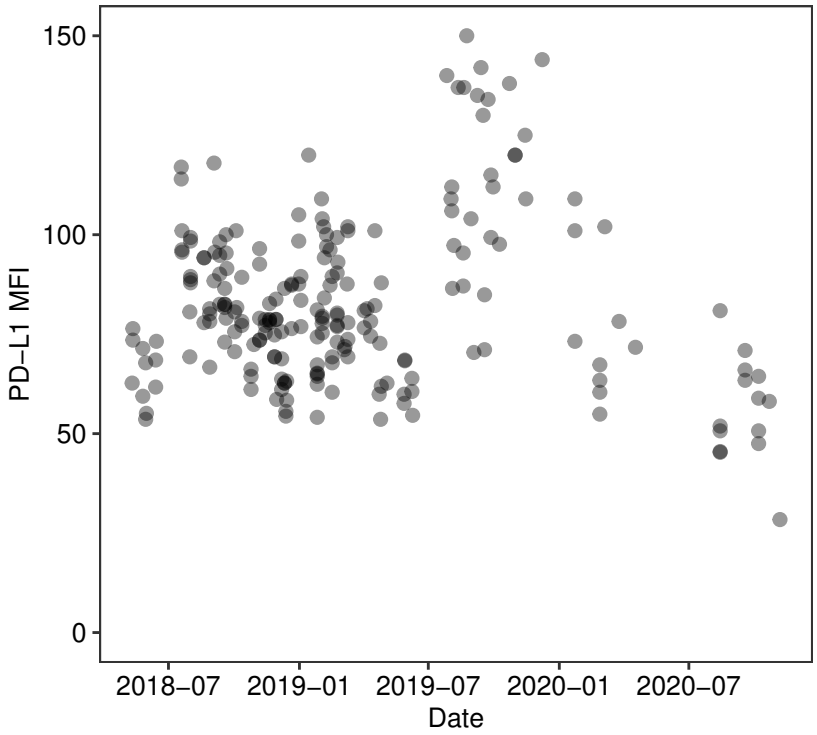

Figure S18: Programmed death-ligand 1 (PD-L1) quality control
PD-L1 expression of PMN- and M-MDSC from cros-sectional and treatment study participants over the course of the study period.

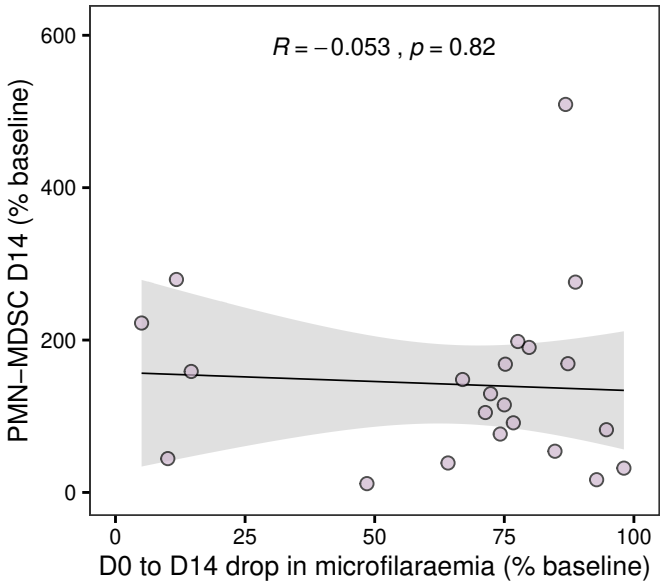

Figure S19: Correlation of change in microfilaraemia and PMN-MDSC numbers
Correlation of percentage of CD66b+ CD14− CD11b+ PMN-MDSC among single PBMC and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4).

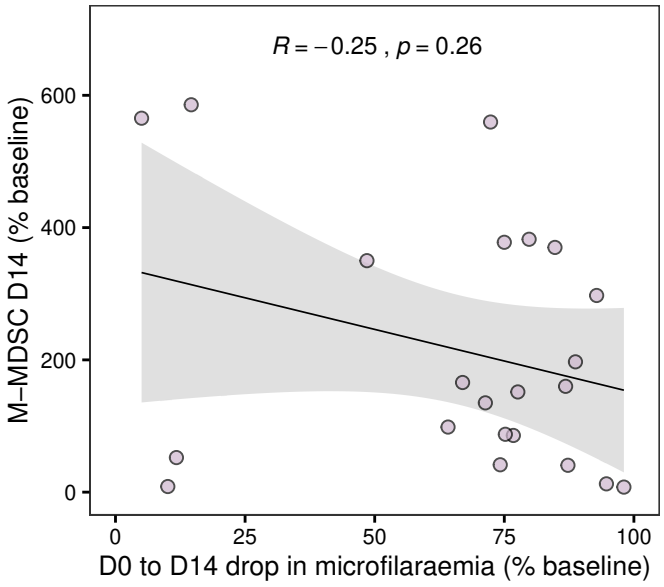

Figure S20: Correlation of change in microfilaraemia and M-MDSC numbers
Correlation of percentage of CD14+ HLA-DR− CD11b+ CD33+ M-MDSC among single PBMC and *Loa loa* parasitaemia on day 14 as percentages of baseline in participants undergoing treatment with albendazole (group 2–4).

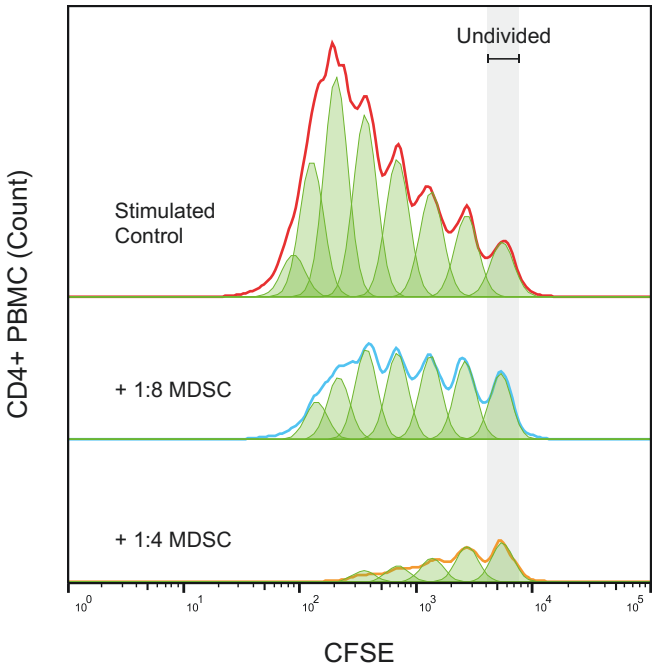

Figure S21: Example of CFSE expression in the T cell proliferation-suppression assay
CFSE expression of stimulated single live CD4+ PBMC from a healthy donor in the absence (control) or presence of augmenting concentrations of PMN-MDSC from a study participant. Green shaded areas represent the cell generations modeled from the measured CFSE intensity.

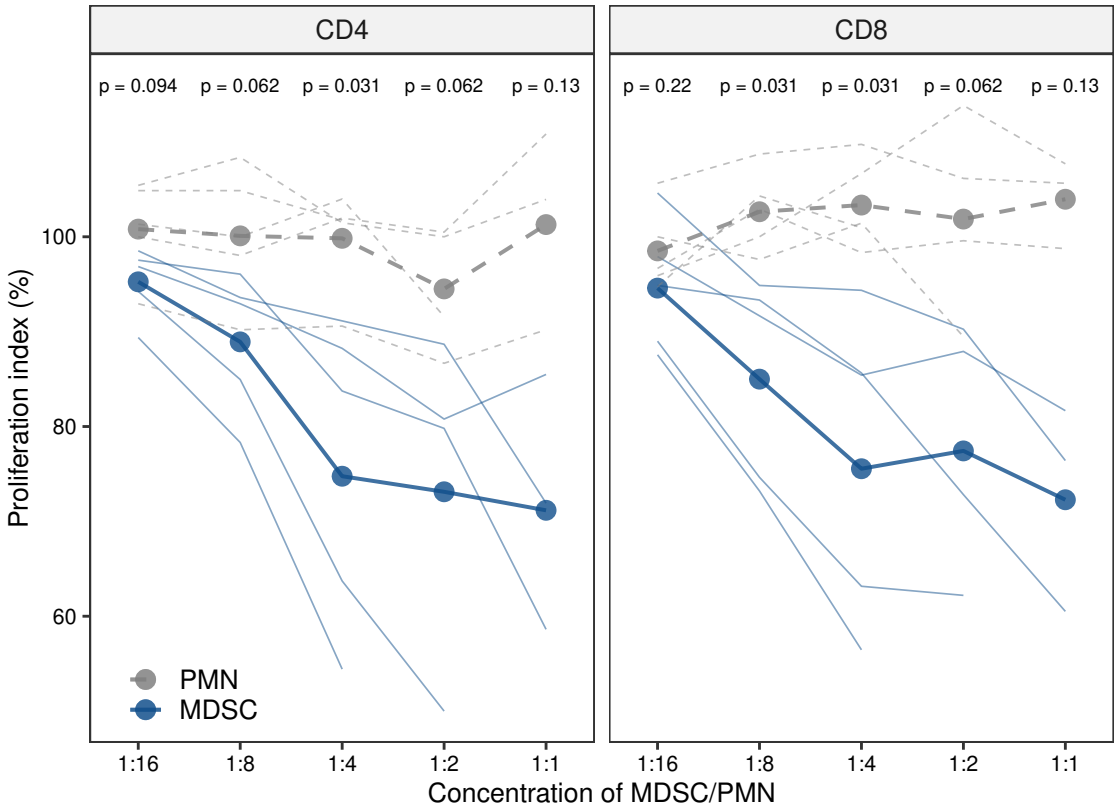

Figure S22: Individual data in the T cell proliferation-suppression assay
Proliferation indices of CD4+ and CD8+ single live CFSE-stained healthy donor peripheral blood mononuclear cells (PBMC) after undergoing a four-day proliferation-suppression assay with increasing ratios of co-cultured polymorphonuclear myeloid-derived suppressor cells (MDSC, blue) or leukocyte controls (PMN, grey) from five study participants. Proliferation indices were normalized to the stimulated control (healthy donor PBMC only). Dots and bold lines represent geometric means and lighter lines represent individual data. One-sided p values were obtained by paired Wilcoxon signed-rank tests.

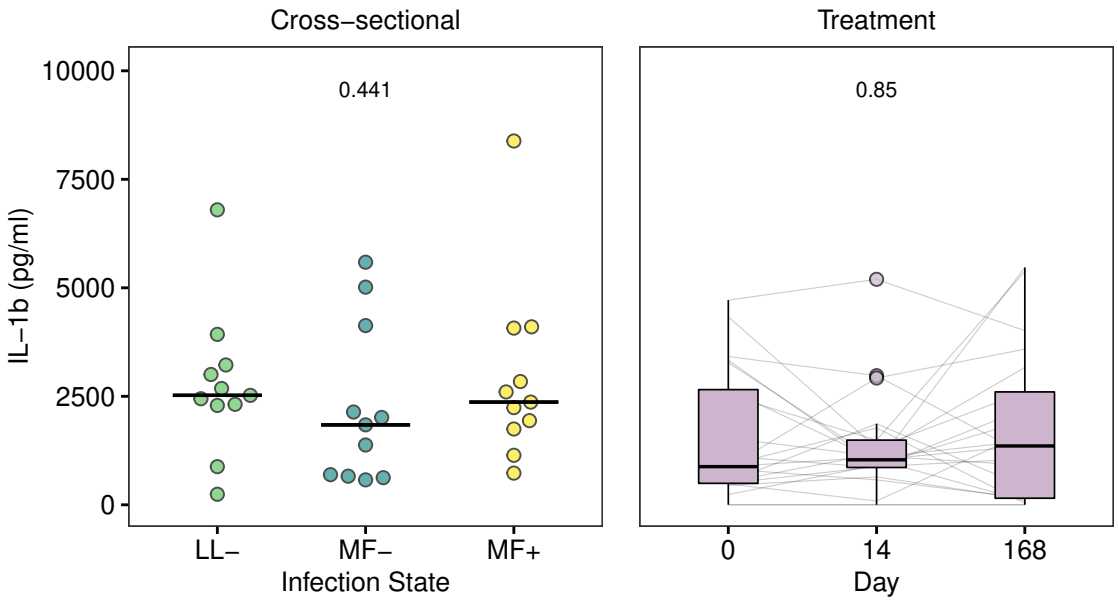

Figure S23: Circulating interleukin-1b
Circulating interleukin-1b (IL-1b) levels in 33 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left panel) and 21 microfilaraemic individuals upon treatment (right panel). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

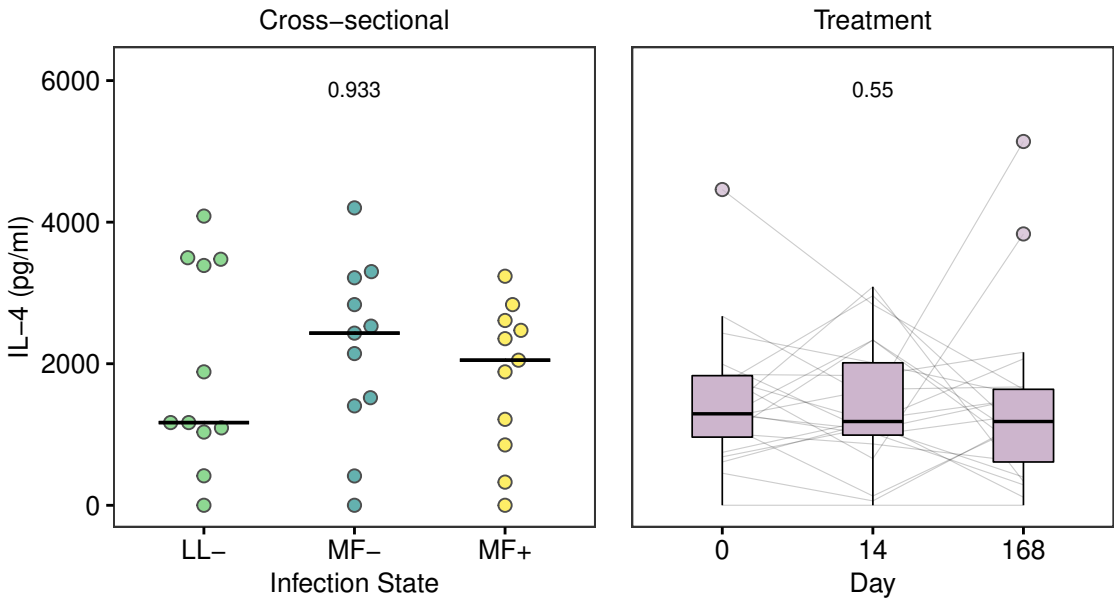

Figure S24: Circulating interleukin-4
Circulating interleukin-4 (IL-4) levels in 33 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left panel) and 21 microfilaraemic individuals upon treatment (right panel). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

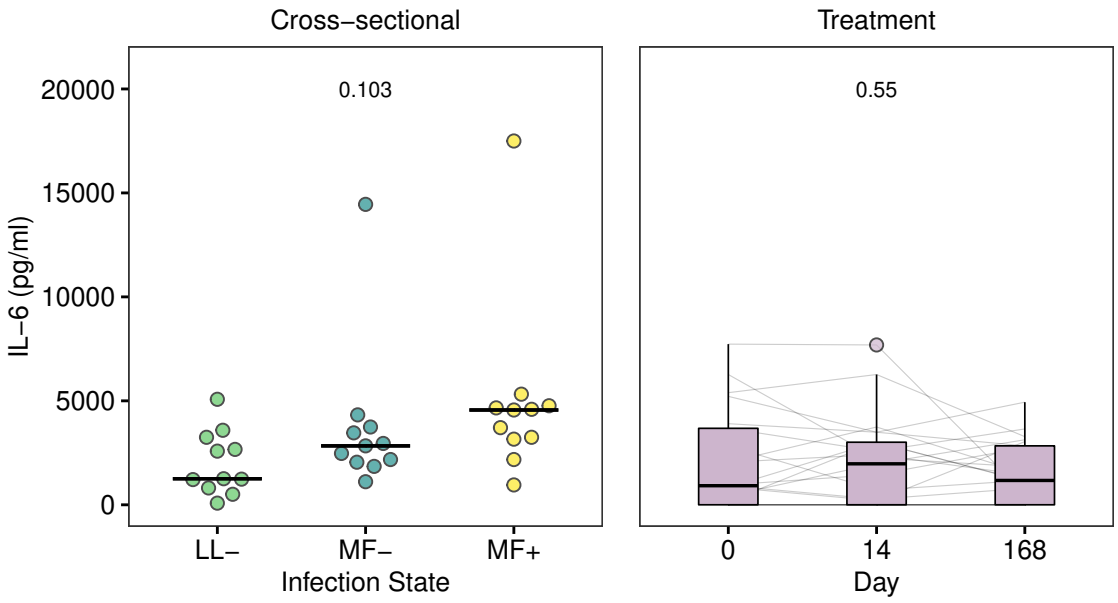

Figure S25: Circulating interleukin-6
Circulating interleukin-6 (IL-6) levels in 33 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left panel) and 21 microfilaraemic individuals upon treatment (right panel). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

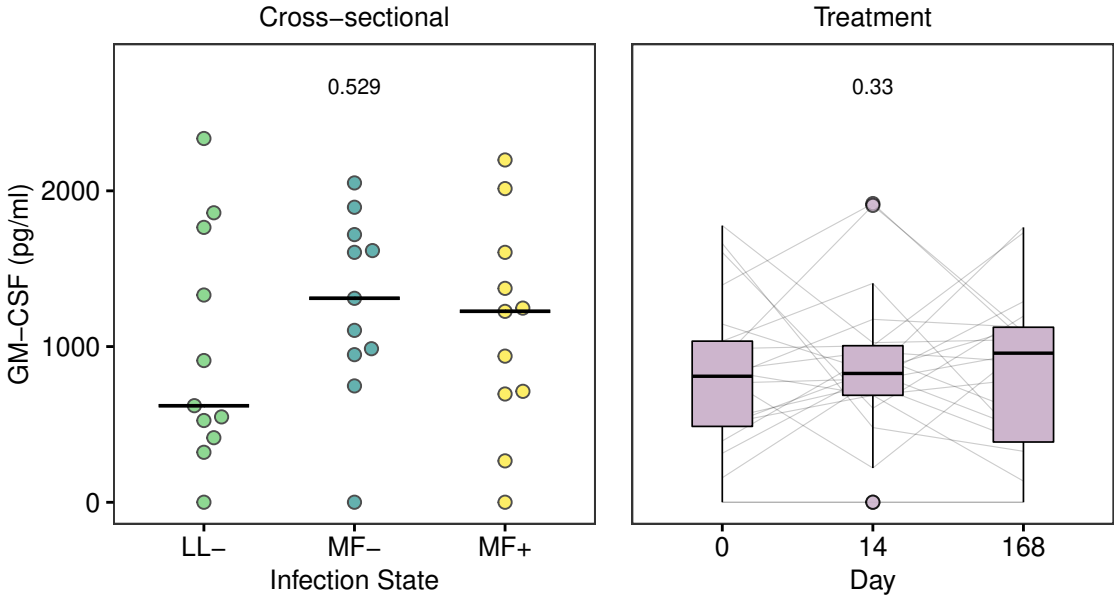

Figure S26: Circulating granulocyte-macrophage colony-stimulating factor
Circulating GM-CSF levels in 33 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left panel) and 21 microfilaraemic individuals upon treatment (right panel). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

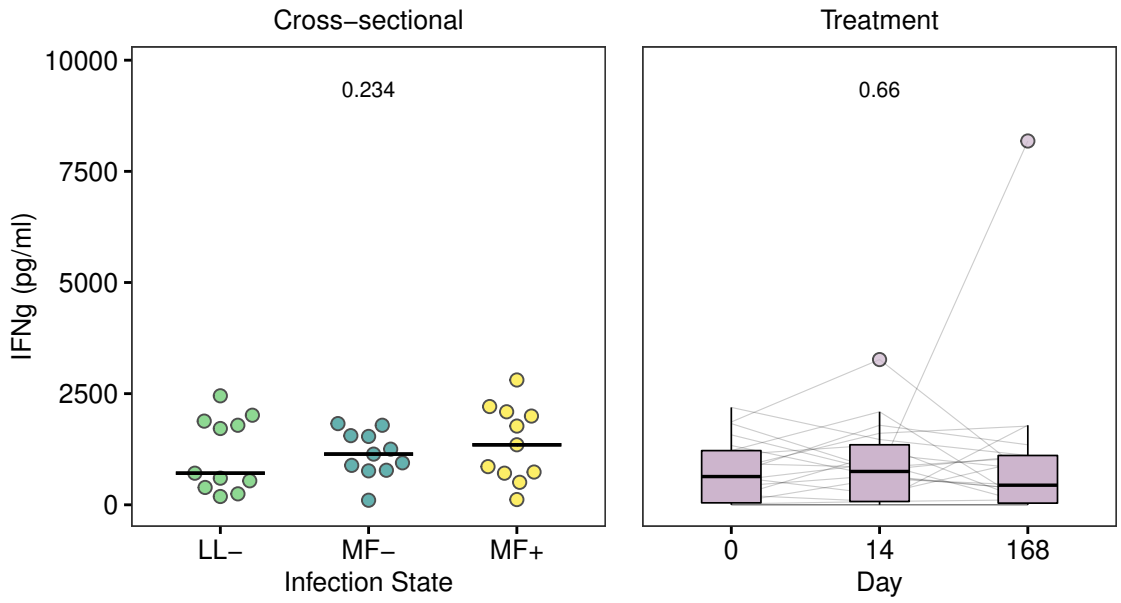

Figure S27: Circulating interferon gamma
Circulating interferon gamma (IFN-γ) levels in 33 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left panel) and 21 microfilaraemic individuals upon treatment (right panel). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

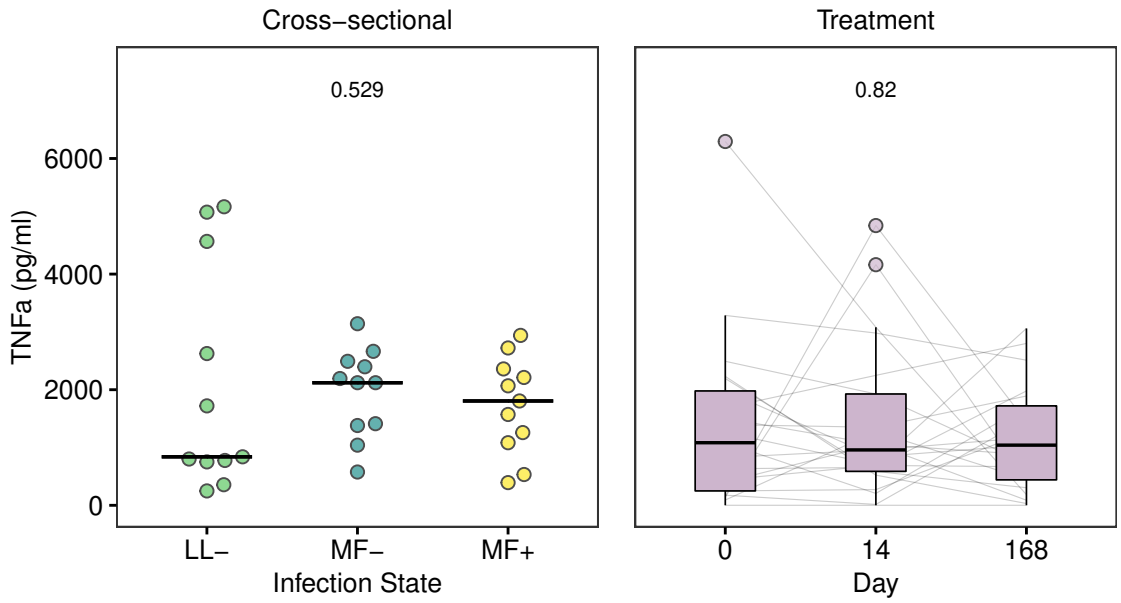

Figure S28: Circulating tumor necrosis factor alpha
Circulating tumor necrosis factor alpha (TNF-α) levels in 33 sex- and age-matched *Loa loa* uninfected (LL-), amicrofilaraemic (MF-) and microfilaraemic (MF+) participants (left panel) and 21 microfilaraemic individuals upon treatment (right panel). Horizontal bars represent the median and boxplots give the median, interquartile range (IQR) and maximum 1.5 IQR. P values were obtained by Friedman's and Nemenyi's post hoc test. Adapted from Burger et al.[235]

10.2 Supplementary Tables

Table S1: Laboratory equipment

Item	Specification	Manufacturer
Micropipette	LABMATE soft, 0.5–10 μl	ABIMED, Langenfeld, Germany
Micropipettes	Finnpipette F2, 1–1,000 μl	Thermo Fisher Scientific, Waltham, USA
Multichannel pipettes	Finnpipette F3, 10–300 μl	Thermo Fisher Scientific, Waltham, USA
Pipette aid	Pipetboy2	Integra, Zizers, Switzerland
Neubauer's counting chamber		R. Langenbrick GmbH, Emmendingen, Germany
Freezing container	Mister Frosty	Thermo Fisher Scientific, Waltham, USA
Centrifuge	Rotanta 460R	Hettich, Tuttlingen, Germany
Microcentrifuge	HERAEUS Pico 17	Thermo Fisher Scientific, Waltham, USA
Freezer	−80 ℃	New Brunswick Scientific, Edison, USA
Freezer	−160 ℃	Sanyo, Osaka, Japan
Fridge	5 ℃	Ocean, Varese, Italy
Vortex	Vortex-Genie 2	Scientific Industries, New York, USA
Biosafety Cabinet Class II	NU-425-600E	NuAire, Plymouth, USA
Precision scale	Kern 440-33N	Kern & Sohn, Balingen, Germany
Water bath	A100	LAUDA, Lauda-Königshofen, Germany
Autoclave	VB-55	Systec, Linden, Germany
CO_2 incubator	HERAcell 150i	Thermo Fisher Scientific, Waltham, USA

Table S2: Laboratory consumables

Item	Specification	Manufacturer
EDTA tube	S-Monovette® 1.2ml K3E	Sarstedt, Nümbrecht, Germany
EDTA tube	S-Monovette® 3.4ml K3E	Sarstedt, Nümbrecht, Germany
EDTA tube	S-Monovette® 7.5ml K3E	Sarstedt, Nümbrecht, Germany
Sodium heparin tube	Conventional closure, glass	BD Biosciences, San Jose, USA
Microscopy slide	76 x 26 mm	R. Langenbrick GmbH, Emmendingen, Germany
Cover slip		R. Langenbrick GmbH, Emmendingen, Germany
Microcentrifuge tubes	1.5 ml	Thermo Fisher Scientific, Waltham, USA
Falcon tube	5, 15, 50 ml	Corning, New York, USA
96-well cell culture plate	polystyrene, U-bottom	Thermo Fisher Scientific, Waltham, USA
Stripettes	1, 5, 10, 25 ml serological pipettes	Corning, New York, USA
Pipette tips	0.5–20 μl	Ratiolab, Dreieich, Germany
Pipette tips	200 μl	Greiner Bio-One, Kremsmünster, Austria
Pipette tips	1,000 μl	Sarstedt, Nümbrecht, Germany

Table S3: Media and solutions

Item	Specification	Manufacturer
Hank's Balanced Salt Solution	1X, calcium, magnesium	Thermo Fisher Scientific, Waltham, USA
RPMI-1640 medium	1X, sodium bicarbonate, without L-glutamine	Sigma-Aldrich, Saint-Louis, USA
RPMI-1640 medium	1X, sodium bicarbonate, without L-glutamine, without phenol red	Merck, Darmstadt, Germany
Ficoll density gradient medium	Ficoll-Paque PLUS	GE Healthcare, Chicago, USA
Phosphate-buffered saline (PBS)	1X, without magnesium and calcium	Thermo Fisher Scientific, Waltham, USA
Fetal bovine serum	sterile-filtered	Thermo Fisher Scientific, Waltham, USA
Giemsa's solution	for microscopy	Merck, Darmstadt, Germany
Giemsa's buffer	phosphate buffer tablets	Merck, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	disodium salt, dihydrate	Sigma-Aldrich, Saint-Louis, USA
Turk's solution		Sigma-Aldrich, Saint-Louis, USA
Dimethyl sulfoxide		Merck, Darmstadt, Germany
Penicillin-Streptomycin-Glutamine	100X	Thermo Fisher Scientific, Waltham, USA
Sodium-Pyruvate	100X	Thermo Fisher Scientific, Waltham, USA

Table S4: Eosinophil/basophil antibody master mix

Volume (μl)	Antibody	Fluorochrome	Specification	Manufacturer
2	CD69	FITC	Clone FN50, mouse IgG1	BioLegend, San Diego, USA
1	CD125	PE	Clone A14, mouse IgG1	BD Biosciences, San Jose, USA
1	CD193	PerCP-Cy5.5	Clone 5E8, mouse IgG2b	BD Biosciences, San Jose, USA
1	CD123	PE-Cy7	Clone 6H6, mouse IgG1	BioLegend, San Diego, USA
1	CD16	APC-Cy7	Clone 3G8, mouse IgG1	BioLegend, San Diego, USA
0.5	CX3CR1	APC	Clone 2A9-1, rat IgG2b	BioLegend, San Diego, USA

Table S5: MDSC antibody master mix

Volume (μl)	Antibody	Fluorochrome	Specification	Manufacturer
8	HLA-DR	PerCP	Clone L234, mouse IgG2a	BD Biosciences, San Jose, USA
1	CD66b	FITC	Clone G10F5, mouse IgM	BD Biosciences, San Jose, USA
1	CD14	APC-Cy7	Clone MφP9, mouse IgG2b	BD Biosciences, San Jose, USA
1	CD11b	APC	Clone M1/70.15, rat IgG2b	Miltenyi Biotec, Bergisch Gladbach, Germany
0.5	CD33	PE	Clone AC104.3E3, mouse IgG1	Miltenyi Biotec, Bergisch Gladbach, Germany
0.5	PD-L1	PE-Cy7	Clone 29E.2A3, mouse IgG2	BioLegend, San Diego, USA

Table S6: Co-Infections with gastrointestinal helminths in treated individuals

	n	% (n=18)
Microscopy positive	7	39
Trichuris trichiura	4	22
Hookworm *spp.*	3	17
Ascaris lumbricoides	1	6
Strongyloides stercoralis	1	6

Baseline stool microscopy results in 18 out of 26 treated individuals in the treatment study. Adapted from Burger et al.[235]

Table S7: Number of participants with adverse events until day 40

Clinical symptom	Control (n = 4)	3 w Alb (n = 9)	5 w Alb (n = 11)	3 w Alb+Ivm (n = 6)	Total (n = 30)	p*
Headache	0	1	3	2	6	0.56
Diarrhea	0	1	3	0	4	1
Vertigo	0	1	3	0	4	1
Fever	0	1	1	1	3	1
Malaria	0	0	1	2	3	1
Back pain	0	1	0	1	2	1
Other	1	2	1	2	6	1
Total	1	5	6	4	16	0.32

* Fisher's exact test between untreated (group 1) and treated (group 2–4) participants